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Biochemical characterization and positional cloning of the var2 variegation mutant of Arabidopsis thaliana

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Biochemical characterization and positional cloning of the *var2* variegation mutant of *Arabidopsis thaliana*

by

Meng Chen

A dissertation submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
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This is to certify that the Doctoral dissertation of

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has met the dissertation requirements of Iowa State University

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TABLE OF CONTENTS

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ABSTRACT

Chloroplasts differentiation and development are highly coordinated with their host cells. To better elucidate the mechanisms involved in nuclear-chloroplast interactions, we characterized a nuclear encoded leaf variegation mutation called *var2*. While cotyledons appear normal, true leaves of *var2* emerge as yellow, then turn into green-white sectors. The green sectors contain cells with normal chloroplasts, whereas the white sectors contain cells with abnormal plastid lacking of internal membrane structures. The phenotypes suggested that VAR2 might be involved in thylakoid biogenesis in early chloroplast differentiation. We cloned the *VAR2* gene by a map-based method. Five original and two potential alleles were sequenced. Deduced amino acid sequence of *VAR2* revealed that it belonged to a gene super family called AAA-ATPase and especially shared high homology with a subfamily of AAA-ATPases called FtsH-like metallopeptidase. VAR2 protein contains three domains including two transmembrane segments on the N-terminus, an AAA-ATPase cassette, and a Zinc binding site for metallopeptidase. Chloroplast import experiments using *in vitro* translated VAR2 protein indicated that VAR2 is localized on the thylakoid membrane with its C-terminus facing the stroma. Northern and Western blots suggested that VAR2 was only expressed in photosynthetic tissues. Both genetic and biochemical analyses suggested that VAR2 forms homo-multimeric complex. The *var2* variegation mechanism is discussed.

CHAPTER 1. GENERAL INTRODUCTION

Dissertation organization

This dissertation consists of two manuscripts. Each manuscript is a chapter (chapter 2-4). There is also a chapter that contains the results of experiments that form the basis of another manuscript. A "general introduction" chapter is at the beginning of the dissertation to introduce background information of the research project; a "summary" chapter is at the end to tie together the work involved in this project.

General introduction

Chloroplasts have been the focus of intense plant biological research. One obvious reason for this is that chloroplasts are specialized organelles for photosynthesis. Another interesting aspect of chloroplasts is that they are semi-autonomous organelles (reviewed in Mullet, 1988; Susek and Chory, 1992; Syndqvist and Ryberg, 1993; Surpin and Chory, 1997). It is well accepted that chloroplasts evolved from an ancient photosynthetic cyanobacterium through a single endosymbiotic event (Morden et al., 1992; Gray, 1993). Even though chloroplasts have their own genomes, more than 80% of chloroplast proteins are encoded in the nuclear genome. The biogenesis of chloroplasts is coordinated with the differentiation and development programs of the host cells. While the molecular mechanisms of photosynthesis are becoming clear, the molecular mechanisms that mediate interactions between chloroplasts and the nucleus are still poorly understood.

Nuclear-chloroplast interactions occur at several levels. First, nuclear genes control the development of chloroplasts. As mentioned above, a large percentage of chloroplast

proteins are encoded in the nuclear genome and transported post-translationally into the chloroplasts. These include chloroplast ribosomal proteins, chloroplast proteins involved in pigment biosynthesis and photosynthesis, and proteins required for the expression of chloroplast genes. Secondly, chloroplasts can send signals to regulate nuclear gene expression according to the status of the chloroplasts. For example, the expression of nuclear encoded *Cab* (chlorophyll a/b binding protein) and *RbcS* (the small subunit of Rubisco) are abolished when chloroplasts are photo-oxidatively damaged (Oelmüller, 1989). Studies on the Arabidopsis *gun* mutants, which have *Cab* and *RbcS* transcripts in the presence of non-functional chloroplasts (Susek et al., 1993), and on the light induction of heat-shock genes (Kropat et al., 1997) suggested that metabolites in the chlorophyll biosynthesis pathway are one kind of chloroplast signal to regulate nuclear gene expression. The aim of my research is to identify nuclear signals or factors that participate in chloroplast development.

Nuclear gene-induced variegation mutants have been used to define nuclear signals that regulate chloroplast development. Variegation mutants usually have green and white (or yellow) leaves. Chloroplasts in the green cells are normal, whereas plastids in the white (or yellow) cells are poorly developed. Nuclear-gene induced variegation mutants can be divided into two groups. In one group, defective plasitids are maternally inherited and in the other group they are not. Most of the well-known nuclear gene-induced variegation mutants have permanently defective plastids that are maternally-inherited. Some examples in this group are the maize *NCS* (nonchromosomal stripe) and *iojap* mutants (Rhoades, 1943; Walbot and Coe, 1979; Newton and Coe, 1986; Coe et al., 1988; Roussell et al., 1991; Han et al., 1992; Gu et al., 1993), the barley *albostrians* mutant (Hess et al., 1992; 1994), and the *Arabidopsis thaliana chloroplast mutator* (*chm*) (Martínez-Zapater et al., 1992). In the case

of *chm* and *NCS*, nuclear mutations induce mutations or rearrangements in the mitochondrial genome, and the mitochondrial mutations affect chloroplast differentiation secondarily to give rise to the variegation phenotype. In both *albostrians* and *iojap*, plastids from white tissue cells are ribosome-deficient and thus permanently-defective. *Ij* has been cloned and sequenced, and codes for a 25kD protein of unknown function (Han et al., 1992).

Although most of the nuclear gene-induced variegations give rise to permanently-defective and maternally-inherited organelles, this is not the case in two Arabidopsis variegation mutants, *immutans* (*im*) (Wetzel et al., 1994) and *var2* (Martínez-Zapater, 1993). In these mutants, the defective plastids are not maternally-inherited, suggesting that they are excluded from reproductive cells or are "cured" during reproduction. The degree of sectoring in *im* is light and temperature sensitive. Phytoene, a noncolored carotenoid precursor, accumulates in the white leaf sectors, indicating that *im* affects the activity of phytoene desaturase (PDS), the enzyme that converts phytoene to ζ-carotene. However, *im* is not the structural gene for PDS (Wetzel et al., 1994), nor does it affect PDS mRNA and protein accumulation (Wetzel and Rodermel, 1998). Recently, *IM* has been cloned by chromosome walking (Wu et al., 1999). The deduced amino acid sequence of *IM* shares a high degree of homology to the mitochrondrial alternative oxidase, but IM is located in the plastid. This suggests that IM might play an important role in quenching excess energy or participating in electron transfer downstream of PDS.

The other Arabidopsis variegation mutant *var2* was first reported and partially characterized by Martínez-Zapater in 1993 as one of only four variegation loci that have been identified in Arabidopsis. The variegation in *var2* is characterized by green and white sectors in leaves, stems, sepals, and siliques, but not cotyledons. Martínez-Zapater reported

that the formation of variegation in *var2* was due to the action of a nuclear recessive gene, and that defective plastids were not maternally-inherited. We have verified Martínez-Zapater's results by doing reciprocal crosses between *var2* and wild-type Col-0. Our data confirmed that F1 progeny, regardless of pollen donor, had a normal phenotype and that *var2* segregates in the F2 progeny in a 3:1 ratio (wild-type : *var2*), as expected of a Mendelian recessive mutation. Hence, the *var2* gene product does not adversely affect plastid or mitochondrial DNA, nor does it lead to permanently-defective organelles. We currently have five alleles of *var2*. These alleles were generated by ethylmethane sulphonate (EMS) or by X-irradiation. The various alleles have visible differences in their degree of white sector formation.

This dissertation presents the characterization of *var2* using molecular, genetic, and biochemical means. The phenotype of *var2* is described in detail in Chapter 2; the molecular cloning of *VAR2* and examination of *VAR2* expression are presented in Chapter 3; and some preliminary biochemical characterizations of the VAR2 protein is presented in Chapter 4.

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CHAPTER 2. THE *YELLOW VARIEGATED* MUTANT OF *ARABIDOPSIS* IS PLASTID AUTONOMOUS AND DELAYED IN CHLOROPLAST BIOGENESIS

A paper published in the *Journal of Heredity*

Meng Chen, Martha Jensen and Steven Rodermel

ABSTRACT

The *yellow variegated* mutant of *Arabidopsis thaliana* is characterized by bright-yellow true leaves that turn green- and white-sectored as leaf development proceeds. Variegation is due to the action of a nuclear recessive gene. Whereas cells in the green sectors contain morphologically normal chloroplasts, cells in the yellow and white sectors are heteroplastidic and contain plastids with rudimentary lamellar structures, as well as some normal-appearing chloroplasts. This indicates that plastids in *yellow variegated* are affected differently by the nuclear mutation (the mutant is "plastid autonomous"). Genetic analyses have revealed that *yellow variegated* is an allele of the *var*2 locus, and that defective plastids are not maternally-inherited. The traits of plastid autonomy and lack of maternal inheritance of the plastid defect set *var*2 apart from other nuclear gene-induced variegations, and define a novel class of variegation mutant. The primary lesion in *var*2 does not likely involve a blockage in the pathways of pigment biosynthesis. Under high temperatures or low light conditions, plant growth is retarded and mutant plants are nearly all-green. Considered together, our data suggest that *var*2 is delayed in chloroplast biogenesis. We suggest that the stochastic pattern of variegation in the mutant may be due to an interplay of factors that regulate *var*2 gene expression and factors that mediate rates of cell and plastid division.

Plastids with a critical threshold of the partially functional *var2* protein are green, while plastids containing less than the threshold of *var2* activity are white.

INTRODUCTION

Chloroplast biogenesis is coordinated with leaf development and involves a complex interplay of exogenous and endogenous factors (reviewed in Mullet 1988; Susek and Chory 1992; Taylor 1989). The signal transduction pathways that regulate chloroplast development, as well as the mechanisms that integrate organelle and nuclear gene expression during this process, are poorly understood. In higher plants, a molecular genetic dissection of nuclear gene-induced variegation mutants is a powerful approach to identify nuclear signals that affect plastid development (e.g., Coe et al. 1988; Giuliano et al. 1993; Gu et al. 1993; Han et al. 1992; Hess et al. 1992, 1994; Martínez-Zapater et al. 1992, 1993; Meehan et al. 1996; Newton and Coe 1986; Rédei 1963, 1967, 1973; Rhoades 1943; Roussell et al. 1991; Walbot and Coe 1979; Wetzel et al. 1994). These mutants have leaves that contain green and white (and/or yellow) sectors. Whereas cells in the green sectors contain morphologically normal chloroplasts, cells in the white sectors generally contain non-pigmented plastids with rudimentary lamellar structures. This indicates that the nuclear gene product defined by the mutation is required for normal chloroplast differentiation.

We have been studying the *immutans* (*im*) variegation mutant of *Arabidopsis* (Meehan et al. 1996; Rédei 1963, 1967; Wetzel et al. 1994; Wetzel and Rodermel 1998). Like many other nuclear gene-induced variegations, *immutans* gives rise to defective plastids in homozygous recessive plants. Unlike these mutants, the plastid defect in *im* is not maternally-inherited. The somatic instability in *im* is modulated by light and temperature,

with white sector formation being promoted by enhanced temperatures, light intensities and/or red light. Phytoene, a noncolored carotenoid precursor, accumulates in white sectors of the mutant, indicating that *im* affects the activity of phytoene desaturase (PDS), the enzyme that converts phytoene to β-carotene. However, *im* is not the structural gene for PDS (Wetzel et al. 1994), nor does it affect PDS mRNA and protein accumulation (Wetzel and Rodermel 1998).

The *yellow variegated* mutant of *A. thaliana* was isolated by Rédei in the 1950's (personal communication). We first became interested in this mutant because of its superficial resemblance to *im*. In this report, we show that *yellow variegated* does not complement *im*, but that it is allelic to the *var*2 locus, first reported by Martínez-Zapater (1993). *var*2 is only one of four nuclear variegation loci that have been described in *A. thaliana* -- the others being *im*, *chloroplast mutator* (Martínez-Zapater et al. 1992; Rédei 1973; Sakamoto et al. 1996) and *var*1 (Martínez-Zapater 1993). Martínez-Zapater (1993) described the gross morphology of mature *var*2 plants and examined the affects of temperature and light on white sector formation. He also mapped *var*2 to chromosome 2. In this report we confirm and extend the observations of Martínez-Zapater, and also examine aspects of the ultrastructure, biochemistry, and molecular biology of *var*2 to gain insight into the primary molecular lesion in the mutant. We conclude that *var*2 is representative of a novel class of nuclear gene-induced variegation mutant.

MATERIALS AND METHODS

Plant material, growth and genetic analyses

Mutant seed stocks were obtained from Dr. G.P. Rédei (University of Missouri), Dr. Jose Martínez-Zapater (Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria, Madrid, Spain), the *Arabidopsis* Biological Resource Center (ABRC), Ohio State University, and from the Nottingham *Arabidopsis* Stock Centre. Wild-type and *var2* plants were maintained in growth chambers at 22^{o}C under continuous illumination (100 μmole $s^{-1}m^{-2}$) using fluorescent and incandescent lights. In some experiments, plants were grown under a 16-hr light: 8 hr dark regime. Complementation tests and analyses of maternal inheritance were performed as described in Wetzel et al. (1994).

Electron and chlorophyll fluorescence microscopy

Samples for electron microscopy included cotyledons and first true leaves of wild type and *var2* plants. Small leaf punches were obtained from green, yellow and white sectors, and the samples were fixed, stained, and examined as described in Horner and Wagner (1980). Phase and chlorophyll fluorescence microscopy were carried out as described in Wetzel et al. (1994). Fresh variegated leaves were placed on slides with water and a cover slip for viewing, and the photographed focal plane was in the mesophyll layer of the leaf. The photographs were taken within 30 minutes of leaf detachment to minimize affects of cell damage.

Pigment analyses

HPLC analyses of chlorophyll and carotenoid pigments were carried out as described in Wetzel et al. (1994). For the determination of total chlorophyll *a* and *b* and carotenoids, pigments were extracted in DMSO at 65°C for 30 min and their concentrations were determined spectrophotometrically (Hiscox and Israelstam 1979; Hodgins and van Huystee 1986).

ALA feeding experiments

δ-ALA feeding experiments were performed essentially as described in Runge et al. (1995). In brief, *var*2 and wild type plants were grown in continuous light in soil. After the seedlings had produced their first pair of true leaves, the plants were gently removed from the soil, the roots were rinsed, and the cotyledons were excised. The plants were then placed in a microfuge tube and vacuum infiltrated with 10mM δ-ALA (pH 7.0) for 1 min, followed by 18 hr in either darkness or light. Pigments were extracted in acetone under a green safelight, and protochlorophyllide concentrations were measured using a fluorometer (excitation 440nm, emission 630nm).

RESULTS

Phenotype of *yellow variegated*

The *yellow variegated* mutant of *A. thaliana* was isolated by Dr. G.P. Rédei (University of Missouri) following X-ray mutagenesis of Columbia seeds (personal communication). The mutant is characterized by variegated stems, true leaves (rosette and cauline), sepals, and siliques. Whereas the cotyledons appear normal, the "yellow" comes from the striking appearance of emerging true leaves, which are bright-yellow. This is illustrated in Figure 1B for a severe allele of *yellow variegated* (described later). Small green islands first appear on the leaves a few days after visible emergence, and as the leaves expand, the yellow color gradually fades out to leave green- and ivory-colored sectors (Figure 1A). The leaves become more green as they expand, but sector boundaries become fixed at full expansion-- i.e., white sectors do not turn green after full expansion is attained. The paired first leaves are always more variegated than the latest leaves to emerge. Leaves that emerge just prior to bolting are often all-green.

Mode of *yellow variegated* inheritance

Rédei found that *yellow variegated* is expressed only in homozygous recessive plants (personal communication). Complementation analyses using a number of "variegated" mutants from the Ohio State and Nottingham Stock Centers revealed that *yellow variegated* is allelic to at least four variegation mutations that have been isolated in *A. thaliana*. These include two alleles of the *var*2 locus, *var*2-1 and *var*2-2 (Martínez-Zapater 1993) (Table 1). In keeping with Martínez-Zapater's nomenclature, we designate the new *var*2 alleles as *var*2-

3, *var*2-4 (*yellow variegated*) and *var*2-5 (Table 1). The phenotype of *var*2-1 is illustrated in Figures 1A and 1B.

Plastids are strictly maternally-inherited in *A. thaliana* (reviewed in Rédei, 1975). To determine whether mutant plastids are maternally-inherited in *yellow variegated*, we reciprocally crossed mutant and wild type plants (Table 2). Variegated reproductive structures were used in the crosses. If *yellow variegated* produced permanently-defective plastids (e.g., due to chloroplast gene mutations), then non-pigmented plastids would be transmitted from variegated females to F1 progeny plants. We observed that all F1 progeny from these crosses, regardless of parentage of the cytoplasm, had a wild-type phenotype. Hence, defective plastids are not maternally-inherited in *yellow variegated.* The data in Table 2 also confirm Rédei's initial observation that *yellow variegated* segregates in the F2 progeny in a 3:1 ratio (wild-type: mutant), as expected of a Mendelian recessive mutation.

_var_2 is delayed in pigment accumulation

The observations in Figures 1A and 1B suggested to us that *var*2 may be delayed in pigment accumulation during leaf and shoot morphogenesis. Consistent with this hypothesis, Figure 2 shows that chlorophyll concentrations, although somewhat reduced in the mutant cotyledons, are dramatically depressed in emerging (yellow) first leaves. Pigment concentrations subsequently increase such that fully-expanded (mature) *var*2 first leaves have nearly normal pigment amounts. This general pattern of pigment accumulation is also true for subsequent leaves. In the wild type, chlorophyll concentrations are reduced in fully-expanded versus young, expanding leaves. This is likely a consequence of senescence-

associated declines in photosynthetic capacity and chlorophyll content that occur during late dicot leaf development in fully-expanded leaves (reviewed in Gepstein 1988).

*var*2-5 plants were used in the analyses in Figure 2 because this mutant is the least severe *var*2 allele (see below) and shows the most dramatic change in chlorophyll concentration as leaf development proceeds. In contrast, the most severe alleles (such as *var*2-1 in Figure 1A) have significantly less pigment than normal in fully-expanded leaves.

_var_2 allelic series

The five alleles of *var*2 have visible differences in their degree of white sector formation. To quantify these differences, we measured the chlorophyll concentrations of just fully-expanded first leaves of the various alleles. The mutants were germinated and maintained under identical conditions in a growth chamber; the seedlings were exposed to continuous illumination. As illustrated in Figure 3, *var*2-1 leaves have significantly less chlorophyll than *var*2-5 first leaves, with the other alleles falling between these two extremes. Therefore, we have classified *var*2-1 as the most severe allele and *var*2-5 as the least severe allele (Table 1).

Is _var_2 blocked in pigment biosynthesis?

One possibility for the delayed accumulation of chlorophyll in *var*2 is that one of the steps of chlorophyll biosynthesis is partially blocked. To address this question we conducted δ-ALA feeding experiments. Feeding δ-ALA, the first committed step in chlorophyll and heme biosynthesis, floods the pathway with precursor, allowing the synthesis of porphyrins to proceed unregulated (Falbel and Staehlin 1994; Granick 1959; von Wettstein 1995). In

wild type plants in the dark, this leads to an accumulation of protochlorophyllide, which can be photoconverted to chlorophyll if the plants are given light. In principle, plants with mutations in chlorophyll biosynthetic genes after δ-ALA formation but prior to protochlorophyllide reduction will accumulate precursors in this part of the pathway, thereby revealing the location of the block; such plants will not accumulate protochlorophyllide in the dark when exogenous δ-ALA is added.

In the case of *var*2, we reasoned that if a step in chlorophyll biosynthesis were inhibited, it might be easiest to detect in emerging yellow leaves of the most severe allele (*var*2-1). As shown in Figure 4A, protochlorophyllide accumulated to similar levels in detached mutant and wild type leaves that were fed δ-ALA in the dark. This indicates that the enzymes between δ-ALA and protochlorophyllide are present and functional at normal levels in the mutant. In contrast, when the leaves were fed δ-ALA in the light, much reduced levels of protochlorophyllide accumulated in the *var*2 and wild type leaves (Figure 4B). This indicates that protochlorophyllide oxidoreductase is functional in young *var*2 leaf tissues.

Although these sorts of experiments cannot pinpoint whether there are defects in the steps of chlorophyll biosynthesis between glutamate and δ-ALA, the data in Figure 4 suggest that the steps of chlorophyll biosynthesis downstream from the dehydratase are not impaired in the mutant. Consistent with this interpretation, HPLC analyses have revealed that pigment precursors do not accumulate in any *var*2 leaf tissues, including yellow first leaves and the white sectors of mature leaves (data not shown). These include the colored chlorophyll and carotenoid intermediates, as well as the noncolored carotenoid precursor, phytoene, which is abundant in *im* white leaf tissues (Wetzel et al. 1994).

The phenotype of *var*2 is plastid autonomous

Figure 1E is a representative light micrograph of a section from an expanding, yellow leaf of *yellow variegated*; Figure 1F is the same section viewed via chlorophyll fluorescence microscopy. A typical cell is outlined. At this stage of mutant leaf development, nascent green sectors first become visible (the intense red chlorophyll autofluorescence), and cells in the yellow part of the leaf are sprinkled with autofluorescing bodies. During later expansion, the leaves become more green and the yellow sectors fade to white. Cells in the white sectors also contain low numbers of autofluorescing bodies, as in Figure 1F (data not shown).

Figure 5A shows normal chloroplasts, with their characteristic grana and starch grains, from an emerging wild type leaf of *yellow variegated*. Morphologically-normal chloroplasts are also found in the green sectors of the mutant (data not shown). Most plastids in emerging yellow leaves lack internal membrane structures (Figure 5B). These plastids are significantly larger than proplastids (3 μm vs. 0.5-1 μm, Bowman 1994), are highly vacuolated, and contain numerous bodies that resemble plastoglobuli, which are lipid droplets that contain quinones, tocopherols and carotenoids (Gunning and Steer 1996; Lichtenthaler 1968). The emerging yellow leaves also contain plastids that appear to be in varying stages of development, ranging from those that are highly vacuolated with rudimentary lamellar structures (Figure 5C, plastid on the left) to those that resemble chloroplasts (Figure 5D, top right). Some cells in the yellow leaves are heteroplastidic and contain plastids with different morphologies (Figures 5C and 5D). This confirms the suggestion from the fluorescence analyses (Figure 1F) that pigmented plastids represent a small fraction of the plastid pool in the yellow sectors. Although heteroplastidic cells are also found in the white sectors of mature leaves, most cells in these sectors are homoplastidic

and contain plastids with a morphology similar to those in Figure 5B-- i.e, highly vacuolated and lacking internal membrane structures (data not shown).

Considered together with the pigment analyses, the fluorescence and ultrastructural analyses suggest that the yellow color of emerging *var*2 leaves may be, in part, an optical illusion caused by green speckles (the red autofluorescing bodies) against a white background. Yet, some of the color may also be contributed by low concentrations of chlorophyll present in plastids with poorly-developed internal membrane structures.

Light and temperature sensitivity of *var*2

To examine whether white sector formation in *var*2 is sensitive to light intensity, we grew mutant and wild type plants on soil at 15 versus 100 μmol $m^{-2}s^{-1}$; the temperature was maintained at 22°C. Figures 1A and 1B show the growth of *var*2-1 (the most severe allele) at the higher light intensity 9 and 18 days after germination. Wild type plants, even though they were green, had a similar number of leaves and a similar morphology under these conditions at each of these timepoints (data not shown). In contrast, Figures 1C and 1D show wild type and *var*2 plants, respectively, at the lower light intensity 30 days after germination. While the two plants are morphologically similar to one another at this light intensity, the *var*2 plants display little variegation at the lower light. We conclude that light intensity is able to influence plant growth rates and the expressivity of variegation in *var*2.

To examine the sensitivity of *var*2 to temperature, we grew *var*2 at 28°C versus 22°C; illumination was maintained at 100 μmol $m^{-2}s^{-1}$. We observed that at both temperatures the plants grew at very similar rates, as monitored by leaf emergence, leaf number, and days to

flowering (data not shown). However, both sets of plants grew more slowly at the higher temperature. Whereas at the lower temperature a significant amount of variegation was observed in the *var*2 plants, at the higher temperature, emerging yellow first leaves of *var*2 had more green sectors, and fully-expanded (mature) leaves were nearly all-green. Some mature leaves were completely green and indistinguishable from wild type leave at this temperature.

We conclude that light and temperature affect the growth rates of wild type and *var*2 *A. thaliana*, and that these factors can also modulate the extent of variegation that occurs in *var*2. This suggests that variegation can be influenced by the rate of plant growth, with green sector formation being promoted by those factors that depress growth rates. Our results are consistent with those of Martínez-Zapater (1993), who reported that light and temperature can influence the extent of sector formation in *var*2. However, he did not examine wild type plants, nor did he monitor plant growth.

DISCUSSION

The *var*2 mutant is "plastid autonomous"

Plastids in *var*2 leaves have different morphologies. Although cells in the white and yellow sectors can be homoplastidic for plastids lacking internal membrane structures, cells in these sectors are frequently heteroplastidic. Heteroplastidic cells usually contain plastids that appear to be in varying stages of thylakoid membrane biogenesis, ranging from those that are highly vacuolated and lacking lamellae, to those that resemble chloroplasts and that probably correspond to the red autofluorescing bodies in Figure 1F. The finding of

heteroplastidic cells indicates that *var2* affects the plastids in a cell unequally and that its expression is "plastid autonomous".

Heteroplastidic "mixed" cells are rare in plants and are thought to be intermediates in a process of sorting-out to form clones (sectors) of cells containing either all-white or all-green plastids (Gillham et al. 1991; Tilney-Bassett 1984). The white plastids in such cells are permanently-defective and thus the plastid defect is maternally-inherited. Permanently-defective plastids in variegated plants can arise either by mutations in the plastid DNA, as in the well-known plastome mutants of *Oenothera* (e.g., Stubbe and Herrmann 1982), or by mutations in the nuclear DNA that permanently affect plastid function. Examples of the latter include the *iojap* mutant of maize (Coe et al. 1988; Han et al. 1992; Rhoades 1943; Walbot and Coe 1979) and the *albostrians* mutant of barley (e.g., Hess et al. 1992, 1994). There are also cases where permanently-defective mitochondria are produced by nuclear recessive gene products that cause rearrangements in the mitochondrial genome. The defective mitochondria are maternally-inherited and secondarily affect plastid function and morphology. Examples of this type of variegation include *chloroplast mutator* in *Arabidopsis* (Martínez-Zapater et al. 1992; Rédei 1973; Sakamoto et al. 1996) and *NCS* (nonchromosomal stripe) in maize (e.g., Gu et al. 1993; Newton and Coe 1986; Roussell et al. 1991).

In contrast to other nuclear gene-induced variegations, defective plastids are not maternally-inherited in *var2*. This suggests that *var2* expression is plastid autonomous due to an 'extrinsic' nuclear-cytoplasmic factor (such as the *var2* gene product itself), rather than to an 'intrinsic' organelle factor that causes a permanent defect (such as an organelle DNA mutation caused by *var2* action). The possibility that plastids can respond differently to

cytoplasmic factors was first suggested in 1943 by Rhoades in his classic studies on *iojap* (Granick 1955; Rhoades 1943). *var2* is thus an ideal system to explore the underlying mechanism(s) of this phenomenon.

Similar to *var2*, *immutans* is plastid autonomous, and defective *im* plastids are not maternally transmitted (Wetzel et al. 1994). *var2*, along with *immutans*, thus defines a novel class of nuclear gene-induced variegation mutant. The lack of maternal inheritance in these mutants may suggest that defective plastids are capable of being restored to normal during or following reproduction. Alternatively, such plastids may be excluded from reproductive cells.

Mechanism of *var2* variegation

The data in this report are consistent with the hypothesis that the *VAR2* gene product is a plastid-localized protein required for normal thylakoid membrane assembly and chloroplast differentiation. We would consequently predict that non-pigmented plastids have less of the protein than chloroplasts. Because *var2* cotyledons appear normal, it is also likely that the *VAR2* gene product first becomes active during the chloroplast differentiation process in true leaves. Based on our interpretation of the different plastid types in the yellow and white sectors of the mutant as intermediates in chloroplast biogenesis, we consider it probable that plastid development is delayed in *var2*. However, we cannot rule out the possibility that some of the plastids in *var2* leaves are photooxidized, or partially so (Meehan et al. 1996; Susek et al. 1993).

Martínez-Zapater (1993) reported that the degree of white sector formation in *var2* could be qualitatively modulated by light and temperature. We have found that both of these

factors also appear to affect plant growth rates. In fact, at either high temperatures or low light conditions, growth rates appear to be retarded and *var*2 leaves are nearly all-green. Therefore, it is possible that the random, non-clonal variegation in *var*2 plants could arise as a consequence of an interplay between 1) factors that determine the temporal and spatial aspects of *VAR*2 gene expression and 2) factors that specify rates of cell and plastid division (increased by high light and low temperature). For example, if *VAR*2 expression were limited to cells in the expanding leaf, enhanced rates of cell and plastid division during the expansion phase could result, in the mutant background, in clones (sectors) of cells, most of whose plastids lacked the necessary amount of this protein for normal chloroplast biogenesis. This assumes that the *var*2 alleles we have examined are not null, and that they produce partially active *var*2 (mutant) proteins.

Regardless of the precise mechanism of *var*2 variegation, an interplay between gene expression and cell division is known to contribute to the specification of developmental patterns in animal species, such as *Drosophila.* A precedent for our working model is also provided by studies on anthocyanin variegation in maize vegetative organs (Cocciolone and Cone 1993; Cone et al. 1993a, 1993b) and mustard cotyledons (Nick et al. 1993). These studies have suggested that there is a stochastic element involved in the light regulation of anthocyanin accumulation, resulting in a patchiness of expression. It has been suggested that this may be due to epigenetic factors (e.g., methylation) and/or to cell-to-cell variations in light perception. Although we cannot rule out similar epigenetic explanations for *var*2 variegation, elements of our working model can be tested, once *var*2 is cloned and sequenced. We are currently using map-based methods to isolate the gene.

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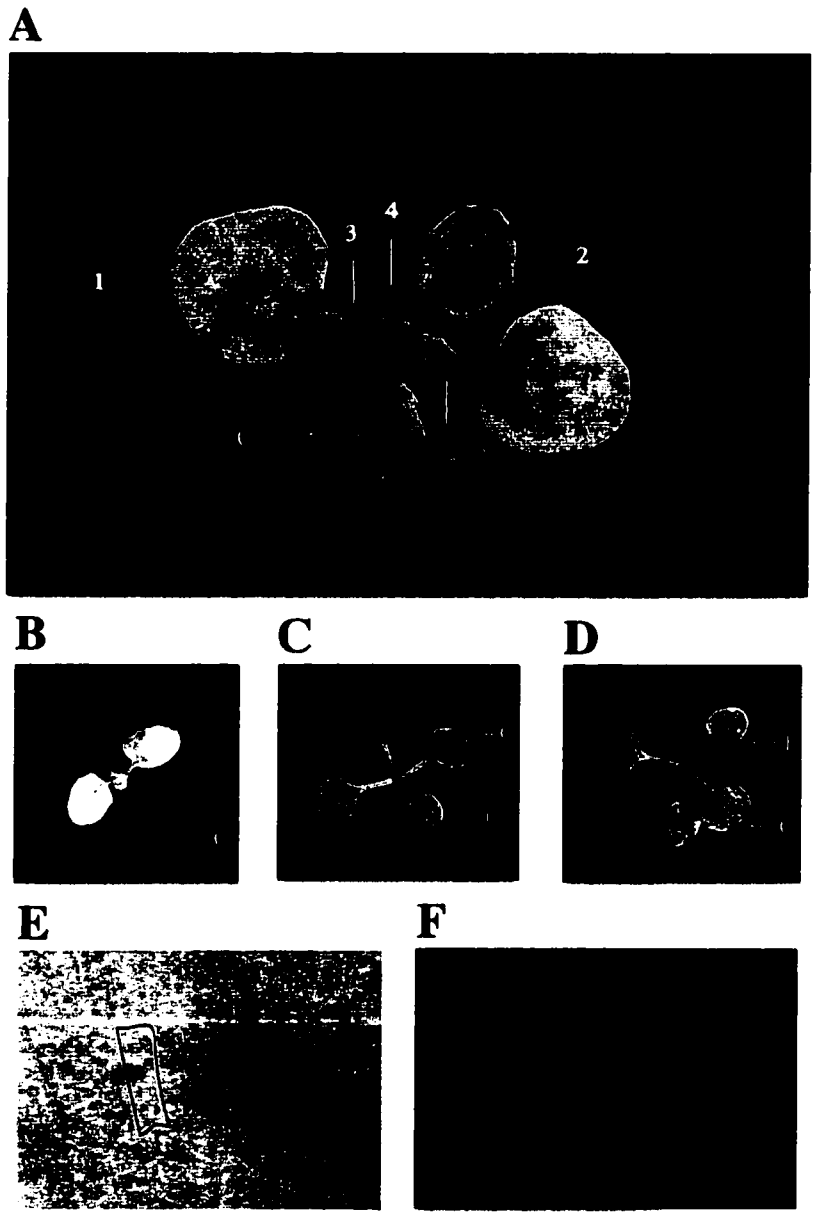

Figure 1. Phenotype of *var2*
A. *var2-1* plants with fully-expanded first leaves 18 days after germination on soil (22°C, 100 µmol $m^{-2}s^{-1}$; 16 hours light/8 hours dark). The order of emergence of the true leaves is shown (paired leaves "1" through "4"). "C", cotyledons. B. *var2-1* with green cotyledons and young, yellow first leaves, 9 days after germination on soil (22°C, 100 µmol $m^{-2}s^{-1}$; 16 hours light/8 hours dark). C. Wild type (Col), 30 days after germination on soil (22°C, 15 µmol $m^{-2}s^{-1}$; 16 hours light/8 hours dark). D. *var2-1*, 30 days after germination on soil (22°C, 15 µmol $m^{-2}s^{-1}$; 16 hours light /8 hours dark). E. Phase micrograph of a portion of a "yellow" first leaf from *var2-4* (*yellow variegated*). A typical cell is outlined. Magnification is 40X. F. Thecorresponding chlorophyll fluorescence image. Plastids with chlorophyll appear red. Magnification is 40X.

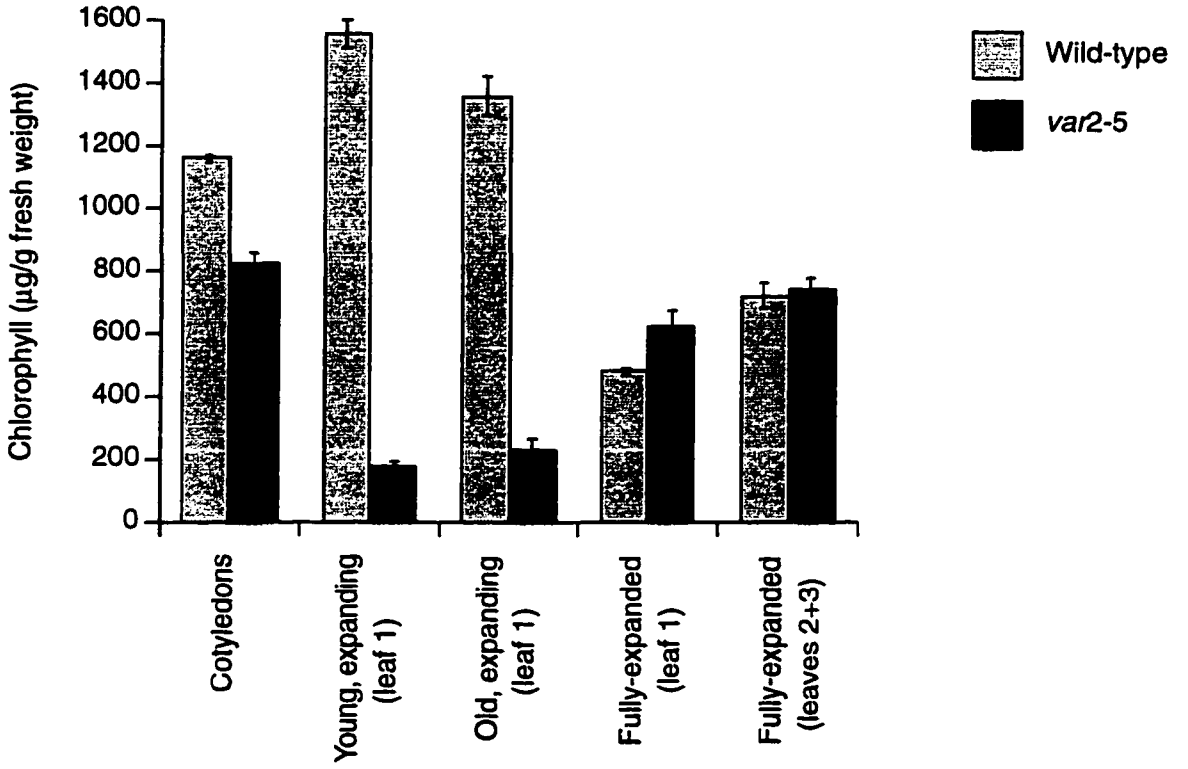

Figure 2. Chlorophyll concentrations during *var*2 development. Chlorophyll determinations were from the cotyledons and first leaves of *var*2-5 and wild type plants. Three stages of first leaf ontogeny were examined: young expanding (yellow), old expanding and fully-expanded. Fully-expanded second and third leaves were also examined. At least five different leaves of each type were sampled (means +/-SE).

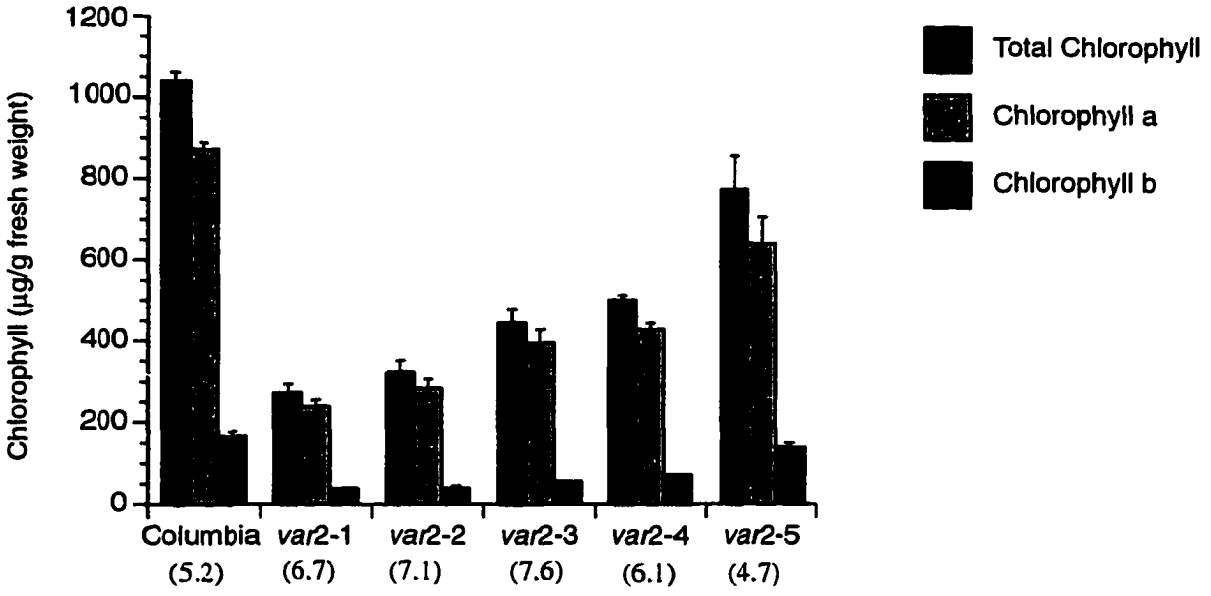

Figure 3. *var2* allelic series. Chlorophyll concentrations were determined on just fully-expanded leaves from wild type and the five *var2* alleles (means +/-SE) growing on soil (22°C, 100 µmol $m^{-2}s^{-1}$; 16 hours light/8 hours dark). At least five different leaves were examined per allele.

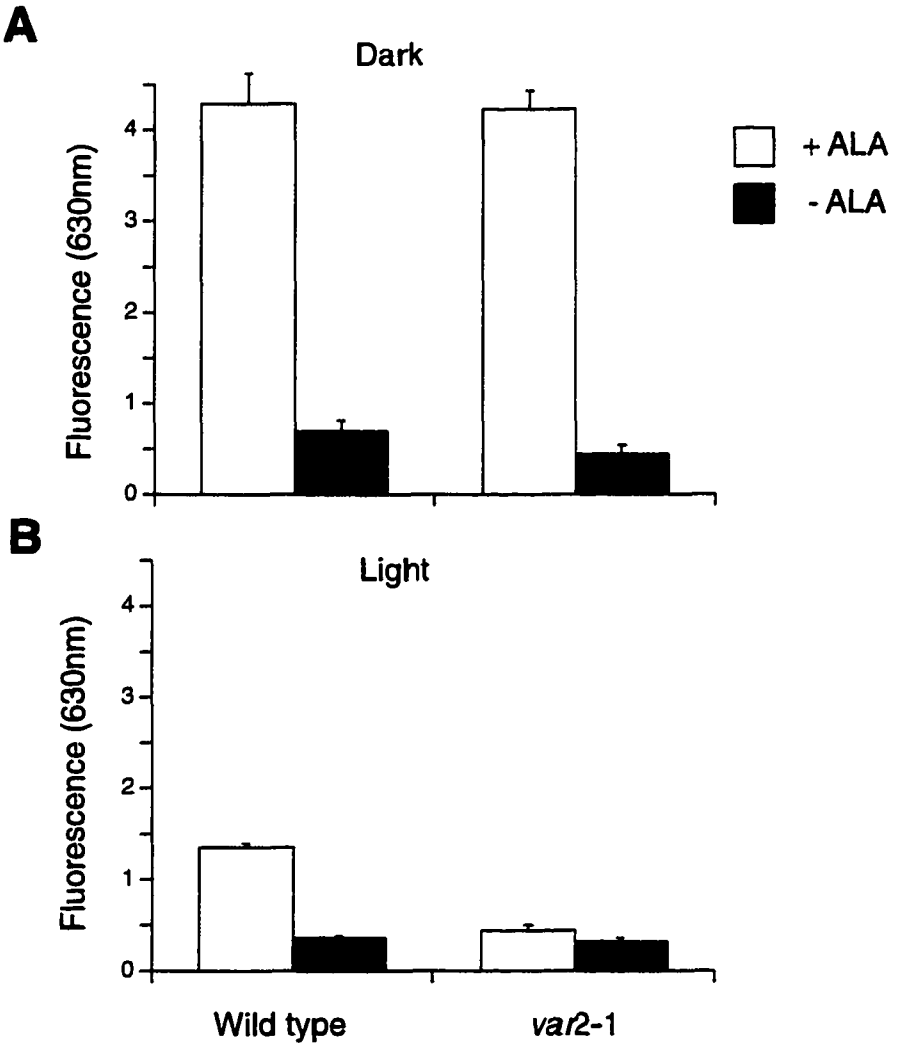

Figure 4. δ-ALA feeding experiment. Detached, first leaves (0.1g) from young *var*2-1 and wild-type plants were fed with 10 mM δ-ALA (pH 7.0) in darkness (A) or light (B) for 18 hrs. The pigments were extracted and protochlorophyllide concentrations were determined by measuring fluorescence at 630nm (excitation 440nm). Each bar represents the means (+/- SE) of at least five separate experiments.

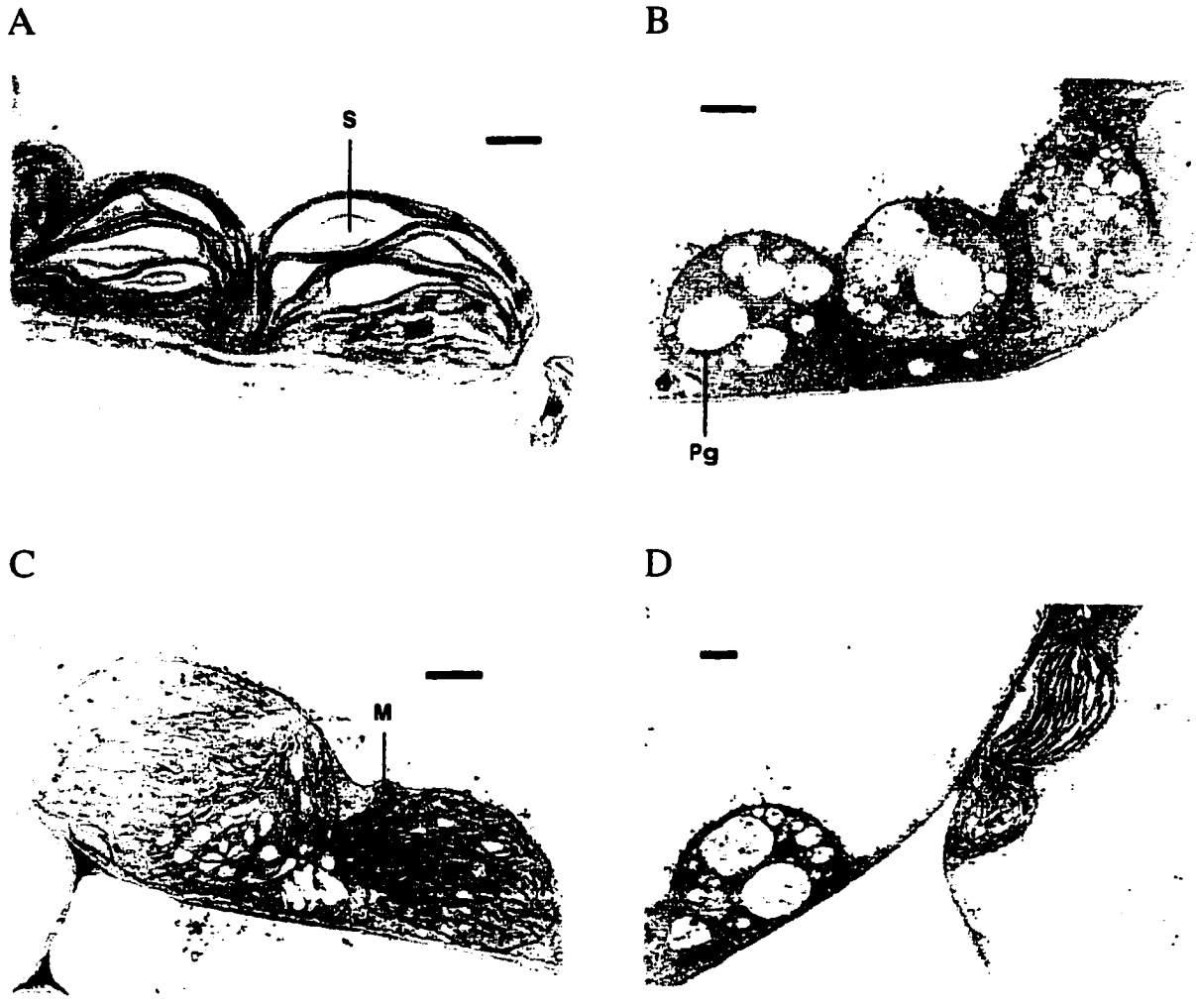

Figure 5. Electron micrographs of plastids from *var*2 leaves. A. A representative chloroplast from wild type first leaves. B.-D., Representative plastids from emerging, yellow first leaves of *var*2-4 (*yellow variegated*). Bar represents 1 μm. "Pg", plastoglobuli. "S", starch. "M", mitochondrion.

CHAPTER 3. AN ARABIDOPSIS VARIEGATION LOCUS DEFINES AN FTSH HOMOLOG OF ENDOSYMBIOTIC ORIGIN THAT FUNCTIONS IN THYLAKOID MEMBRANE BIOGENESIS

A paper submitted to the *Plant Cell*

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ABSTRACT

The Arabidopsis *var*2 locus conditions the formation of variegated plants. Cells in the green sectors contain normal chloroplasts, whereas cells in the white and yellow sectors contain plastids arrested in the early stages of chloroplast biogenesis. We have positionally cloned *VAR*2 and discovered that it encodes a homolog of FtsH, an ATP-dependent metalloprotease implicated in a variety of membrane biogenic processes in prokaryotes and eukaryotes. We show that VAR2 is of endosymbiotic origin and that it functions in the biogenesis of thylakoid membranes. This is the first *in vivo* evidence that FtsH is active in membrane biogenesis in a multicellular organism. Expression of VAR2 in *E. coli* gives rise to the well-known filamentation temperature sensitive (*fts*) phenotype, suggesting that VAR2 and FtsH have conserved activities. We have characterized four *var*2 alleles and two

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approximate the null phenotype. We conclude that the mechanism of *var2* variegation involves the action of a compensating activity.

INTRODUCTION

Chloroplasts are derived from undifferentiated proplastids early in leaf development (reviewed in Mullet, 1988; Leon et al., 1998). A major part of this developmental process involves the formation of photosynthetically-competent thylakoids, which comprise the internal membrane system of chloroplasts. Thylakoids are organized into either granal stacks (enriched in PSII) or as extended stromal lamellae (enriched in PSI) (Green and Durnford, 1996). Plastids are bounded by an envelope composed of an inner and an outer membrane, and a variety of ultrastructural, biochemical and molecular analyses have indicated that thylakoids originate from proplastid inner membranes by invagination and vesicle formation (Morré 1991 a, b; Hoober et al., 1994; von Wettstein, 1995). Thylakoids may also be maintained throughout the life of the chloroplast by fission/fusion events involving the inner membrane, because many of the pigments, proteins and lipids characteristic of mature chloroplasts are found in both membranes (Reinbothe and Reinbothe, 1996; Joyard et al., 1998; Zerges and Rochaix, 1998). By analogy to the secretory system (Farquhar and Palade, 1998), it is possible that thylakoid membrane function further involves retrograde vesicle traffic from thylakoids to the inner membrane. Although evidence for this sort of trafficking is lacking, it would provide a mechanism for the dispersal of thylakoids, e.g., such as occurs during fruit development, when chloroplasts differentiate into carotenoid-accumulating chromoplasts (e.g., Camara and Brangeon, 1981). The mechanisms of thylakoid biogenesis, maintenance and disassembly are poorly understood.

To gain insight into the factors that regulate chloroplast biogenesis, we have been studying nuclear gene-induced variegation mutants of Arabidopsis (Wetzel et al., 1994; Meehan et al., 1996; Wetzel and Rodermel, 1988; Chen et al., 1999; Wu et al., 1999). One such mutant, *var2*, was intriguing to us because it appeared to be delayed in chloroplast development (Figure 1A) (Martínez-Zapater, 1992; Chen et al., 1999). Cotyledons in *var2* appear normal, but the first true leaves are bright yellow. As leaf expansion proceeds, green islands become visible and expand in size, and the yellow sectors fade to white. Sector boundaries and identity become fixed at full expansion. With the exception of cotyledons, all normally-green structures of *var2* are variegated. Sector formation in *var2* is generally insensitive to growth illumination, except at very low intensities, when nearly all-green, small plants are formed.

Whereas cells in the green sectors of *var2* contain morphologically normal chloroplasts, cells in the yellow and white sectors are heteroplastidic and contain a variety of plastid types (Chen et al., 1999). These include plastids with large vacuoles and no organized internal lamellar structures, plastids with rudimentary lamellae, and normal-appearing chloroplasts. Heteroplastidic cells are rare in plants because plastid types normally "sort out" to form clones of cells containing one plastid type or the other (Tilney-Bassett, 1975). The presence of "mixed" cells in *var2* indicates that the mutant is "plastid autonomous", i.e., that *var2* plastids are affected differently by the nuclear mutation. The defective, white plastids are not maternally-inherited in *var2* (Chen et al., 1999). This is in contrast to most variegations, which give rise to permanent plastid defects (Tilney-Bassett, 1975), and suggests that the defective *var2* plastids are either restored to normal during reproduction or are excluded from reproductive cells. Biochemical assays showed that

carotenoid and chlorophyll precursors do not accumulate in *var*2, and that the mutant is not blocked in the steps of pigment biosynthesis (Chen et al., 1999).

In this study, we report the positional cloning of *VAR*2, and we define the nature of four *var*2 alleles produced by X-ray- and EMS-mutagenesis. We show that VAR2 bears high similarity to the FtsH subfamily of AAA proteins. FtsH is an ATP-dependent metalloprotease that mediates a variety of membrane-associated events in prokaryotes and yeast (reviewed in Suzuki et al., 1997). FtsH homologs have also been identified in multicellular organisms (Beyer et al., 1997), but these proteins are poorly characterized. *var*2 is the first FtsH mutant reported in a multicellular organism, and our analyses provide *in vivo* evidence that VAR2 functions during the early steps of thylakoid membrane biogenesis.

RESULTS

Ultrastructural analyses of *var*2

We have previously reported that cells in the green sectors and cotyledons of *var*2 contain normal-appearing chloroplasts (Figure 1B), and that cells in the white (and yellow) sectors are heteroplastidic (Figure 1C) (Chen et al., 1999). All of the different plastid types in *var*2 leaves are the size of normal chloroplasts (~3 um diameter) and are much larger in size than proplastids (~0.5 um diameter; Bowman, 1994). Plastids in dark-grown *var*2 seedlings (etioplasts) resemble those in the wild type and contain normal-appearing prolamellar bodies (PLB) (Figure 1D). Upon illumination, etioplasts differentiate into chloroplasts and the PLBs disperse to form typical thylakoids (reviewed in von Wettstein,

1995). Mitochondria in cells of the white/yellow sectors of *var*2 have a normal appearance (data not shown).

Positional cloning of *var*2

*var*2 maps to chromosome 2 (Martínez-Zapater, 1992). To initiate a chromosome walk to the gene, we generated an F2 mapping population by crossing *var*2-4 (in a Columbia ecotype background) (Chen et al., 1999) with Landsberg *erecta*. *var*2 mapped between cleaved amplified polymorphic sequence (CAPS) marker m283 and a CAPS marker generated to *COP*1. Because m283 resides within a YAC contig in this region (Zachgo et al., 1996), we oriented the YACS with respect to *var*2 using CAPS markers generated to YAC end clones; four of these YACs are illustrated in Figure 2A. While these procedures were in progress, we found that SSLP marker nga361 mapped very close to *var*2 and that it hybridized to two of the YACs (yUP11D1 and CIC11E1) in the contig. We also observed that it hybridized to two bacterial artificial chromosomes (BACs) (TAMU8A11 and TAMU2B11). CAPS markers were generated to the ends of BAC TAMU8A11 and the right end (designated 8A11R) was found to co-segregate with *var*2. 8A11R was used as a probe to isolate a series of overlapping cosmid (pOCA8A11R) and plasmid clones (pMC1, pMC2 and pMC3) bearing sequences from the two BACs. The cosmids and plasmids failed to overlap an ~5 kbp region, and a PCR product (pMC4) was generated to span the gap. *var*2 was finally mapped between pOCA-MC1 (a CAPS marker derived from cosmid pMC1) and nga361, an ~20 kbp interval.

To localize *VAR*2 within this interval, we subcloned fragments of pMC3 (which nearly spans the interval) and used them to probe an *Arabidopsis* cDNA library (Newman et

al., 1994). This library represents RNAs expressed from a variety of *Arabidopsis* developmental stages, including early leaf development when we would anticipate *VAR2* to be expressed (Chen et al., 1999). cDNAs from three different genes were identified (ORF1, ORF2 and ORF4). An additional ORF (designated ORF 3) was found to reside in pMC3 after sequencing an end of pOCA8A11R. This cosmid end was found to contain a partial ORF (i.e., ORF 3), and the size of this ORF was extended by additional sequencing.

Primers were designed to sequences in each of the four ORFS to amplify the corresponding genomic DNA fragments from Columbia and four different *var2* alleles, all in a Col-0 background: *var2*-1, *var2*-2, *var2*-3, and *var2*-5 (Chen et al., 1999). Sequences of the amplification products were determined by primer walking. Whereas sequences encompassing and flanking ORFS 1, 2 and 3 were identical among the strains tested, there were mutations in ORF 4 in each of the four *var2* alleles (Figure 2B). We thereby designated ORF4 as *VAR2*.

During the cDNA library screening, eight *VAR2* cDNAs were isolated. These cDNAs differed in the lengths of their 5'-ends, but otherwise were identical in sequence. This suggested that *VAR2* was a single-copy gene in Arabidopsis, consistent with low-stringency genomic DNA gel blot analyses (data not shown). The longest of the eight cDNAs was 2.28 kb. This was the size of *var2* mRNAs estimated by RNA gel blot analysis (Figure 2C). The northern hybridization experiments also demonstrated that *var2*-3 and *var2*-5 have normal transcript levels, but that *var2*-2 and *var2*-1 mRNAs are reduced in abundance compared to the wild type (35% and 12% of normal VAR2 mRNA levels, respectively).

Comparison of the cDNA and genomic sequences revealed that VAR2 contains five exons. The first in-frame ATG is at the beginning of the second exon, and we assume that it

represents the start codon because the size of the 2.28 kb cDNA corresponds well with the size of the transcript estimated by northern blot analysis (Figure 2C). *VAR2* codes for a protein with a molecular mass of 74.15 kD (696 amino acids) and a pI of 5.99. Based on the predicted amino acid sequence, three of the *var2* alleles are missense mutations--*var2*-2 (R191K), *var2*-3 (G267D) and *var2*-5 (P320L)-- and *var2*-1 is a nonsense mutation (Q597*) (Figure 2B).

In summary, we conclude that we have isolated *VAR2* on the basis of the fine-mapping studies, the identification of mutations in a gene from four independent *var2* alleles, and marked alterations in mRNA expression levels from two of the four alleles.

VAR2 is an FtsH homolog

The *VAR2* gene product bears high similarity to the AAA protein class of Walker A/GTPases (Walker et al., 1982; Schulz, 1992). AAA proteins ("ATPases associated with diverse cellular activities") are ubiquitous among prokaryotes and eukaryotes, and they have in common an ~200-250 amino acid "AAA cassette" (Kunau et al., 1993). This cassette contains an ATP binding site that includes Walker boxes A and B, as well as other conserved domains whose functions are not understood (Smith and Rayment, 1996; Beyer, 1997). AAA protein subfamilies have been identified, and these differ from one another in the number and structure of their AAA cassettes, and by the presence of other distinguishing sequence motifs.

VAR2 shows high similarity to the "metal-dependent protease" AAA protein subfamily (Figure 3). The members of this family contain a single AAA cassette, a putative zinc-binding domain in their C-termini, and two transmembrane helices in their N-termini

(Beyer, 1997) (Figure 3A). In Figure 3B, the cDNA sequence of *VAR2* is compared to FtsH sequences from *E. coli* (Tomoyasu et al., 1993a), red pepper (Hugueney et al., 1995) and Arabidopsis (Lindahl et al., 1996). Among the four sequences, the AAA cassette and putative Zinc-binding domains are highly conserved. However, the transmembrane domains are less well conserved, and it was not possible to align the C-termini because they bear little overall resemblance. We predict that *var*2-3 and *var*2-5 have missense mutations in the ATP binding site, and that *var*2-2 has a missense mutation at the end of the second transmembrane domain (Figure 3A). The nonsense mutation in *var*2-1, on the other hand, occurs at the extreme C-terminus; a truncated protein, if stable, would contain all of the conserved motifs.

Current evidence favors the view that modern day plastids are derived from a single cyanobacterial-like endosymbiont (monophyletic origin hypothesis), and that during the course of evolution, most of the genome of the symbiont was transferred to that of the host, perhaps to consolidate control of gene expression within the nucleus-cytosol (reviewed by Doolittle, 1998; Martin and Herrmann, 1998). Although the evolutionary history of the metal-dependent protease class of AAA proteins is not well understood, they appear to be derived from a prokaryotic *FtsH* gene (Beyer, 1997). Among photosynthetic organisms, four FtsH homologs have been identified in the completely sequenced genome of the cyanobacterium *Synechocystis* 6803 (Kaneko et al., 1996). They have also been found in the nuclear genomes of higher plants and in the plastid genomes of red algae (*Cyanidium*) and brown algae (*Porphyra*) (Reith, 1995). As illustrated in Figure 3C, *FtsH*-like genes fall into two clearly defined lineages (100% bootstrap support). Consistent with an endosymbiotic origin, nuclear *FtsH*-like genes in both lineages 1 and 2 are similar to cyanobacterial FtsH homologs. After the ancestor of the cyanobacterial homolog slr1604 was transferred to the

nuclear genome (lineage 1), it likely duplicated, giving rise to Arabidopsis *FtsH* 1 and *FtsH* 2. *VAR2* is in a different lineage and appears to have arisen by gene transfer of a different cyanobacterial homolog (an ancestor of slr0228 and slr1309). The transfer of cyanobacterial *FtsH* homologs to the nucleus was not a universal occurrence, because modern-day *FtsH* is present as a single copy gene in the plastid genomes of red and brown algae.

VAR2 is a thylakoid membrane protein

To localize VAR2, we performed *in organello* import experiments using isolated pea chloroplasts (Figure 4). We found that the labeled protein is imported into chloroplasts, where it is ~5 kD smaller than the protein transcribed and translated in vitro ("CT" versus "TL" lanes). This is consistent with predictions using the ChloroP software program (Emanuelsson et al., 1999) that VAR2 contains an ~5kD N-terminal chloroplast targeting sequence with a cleavage site between amino acids 47 and 48 of the precursor.

Following fractionation of chloroplasts on a sucrose step gradient, we found that VAR2 is localized in the thylakoid membrane fraction ("T") and is not detectable in the envelope ("E") or stroma ("S"), even after prolonged exposure of the fluorographs. As a control, we observed that imported light harvesting chlorophyll *a/b*-binding (CAB) proteins of photosystem II (Green and Durnford, 1996) were localized exclusively in the thylakoid fraction, as expected (data not shown). VAR2 is not removed from the thylakoids by NaOH ("T + NaOH"), indicating that it is an integral membrane protein. VAR2 is also sensitive to trypsin digestion ("T + TRY"). This would be expected if the large hydrophilic C-terminus, which contains the putative ATP and Zn^{+2}-binding domains (Figure 3A), is on the outside of the isolated thylakoids (i.e., exposed to the stroma in vivo).

The topography of VAR2 is similar to that of the *E. coli* FtsH protein, with the cytoplasm being analogous to the stroma and the periplasmic space being analogous to the thylakoid lumen (Ogura et al., 1991; Tomoyasu et al., 1993 b). It is also similar to the topography established for the Arabidopsis "FtsH1" protein (Lindahl et al., 1996).

VAR2 mRNA expression

RNA gel blot analyses were performed to examine the expression of *VAR2*. Figure 5A shows that *VAR2* transcripts are abundant in all normally green organs of the plant, including mature leaves, cauline leaves, cotyledons and very young siliques. *VAR2* mRNAs accumulate to low levels in stems, and are undetectable in roots. It is likely that the small amount of *VAR2* transcripts in flowers is derived from the sepals. Figure 5B shows that little *VAR2* transcript accumulation occurs in dark-grown seedlings, but that *VAR2* mRNAs are induced by light, with about 20% of normal levels accumulating after 12 hours of illumination.

VAR2 expression in *E. coli* gives rise to a *fts* phenotype

Mutations in *FtsH* mediate a variety of membrane-associated phenotypes in *E. coli*. Null alleles are lethal and overexpression is deleterious to cell growth (Ogura et al., 1991). *FtsH* mutants were originally isolated as cell division mutants, and depending on the background *E. coli* strain, they display either a filamentation temperature sensitive (*fts*) phenotype (failure to septate at 42°C but not at 30°C) or a thermosensitive growth phenotype (an immediate cessation of growth at the higher temperature) (e.g., Ogura et al., 1991;

Granger et al., 1998). Although the mechanism is not clear, these mutations are thought to cause a defect in a membrane-associated event(s) required for normal cell division.

When mutant forms of FtsH are expressed from a plasmid in *E. coli.*, mutations in the N-terminus between the two transmembrane domains (i.e., the domain in the periplasmic space) are recessive and do not affect cell division, whereas mutations in the C-terminal cytoplasmic domain give rise to cells with an *fts* phenotype (Akiyama et al., 1994b). FtsH is a homodimer, and the recessive phenotypes are thought to arise because mutations in the periplasmic space domain prevent dimer formation (Ogura et al., 1991; Tomoyasu et al., 1993b; Akiyama et al., 1994b; 1995; 1998); hence, only dimers composed of normal FtsH subunits (i.e., coded for by the chromosomal DNA) can form. Mutations in the C-terminus, on the other hand, are thought to affect the functionality of dimers, rather than dimer formation; hence, a dimer containing a mutant and normal subunit is nonfunctional, giving rise to the dominant negative *fts* phenotype.

We introduced *VAR2* into *E. coli* and observed that the cells formed long filaments and had decreased rates of cell division at 42°C but not 30°C (Figures 6D versus 6C). Control transformations with pBluescript expressing _-galactosidase gave rise to cells with normal division rates at both temperatures (Figures 6A and 6B). We conclude that *VAR2* expression gives rise to an *fts* (dominant negative) phenotype. VAR2 and *E. coli* FtsH may thus have similar activities. Analogous to the situation in *E. coli*, one possibility is that VAR2 is capable of forming dimers with *E. coli* FtsH, but that once formed, VAR2 poisons dimer function. Our results are similar to those observed when the *Helicobacter pylori* FtsH protein is expressed from a plasmid in *E. coli* (Ge and Taylor, 1996).

DISCUSSION

FtsH function in prokaryotes and eukaryotes

The function of FtsH has been most fully characterized in *E. coli*, where a number of diverse activities have been identified. One of its primary roles is in protein quality control, where it degrades excess subunits of soluble and membrane-bound proteins (reviewed in Suzuki et al., 1997; Gottesman et al., 1997). These include SecY (a component of the protein translocase), heat-shock transcription factor σ^{32}, the "a" subunit of the F_1F_0 ATPase (F_0a), and cII, a transcription factor that mediates the ysogeny/lysis decision. FtsH is also involved in the integration of membrane proteins, protein export from the cell, mRNA decay, and resistance to colicins (e.g., Akiyama et al., 1994a, Suzuki et al., 1997, Granger et al., 1998). It has been proposed that the versatility of FtsH is related to the fact that it possesses both chaperone and protease activities (e.g., Akiyama et al., 1994a; Shirai et al., 1996).

The function of FtsH in eukaryotes is poorly understood. Several FtsH homologs have been identified in yeast (reviewed in Suzuki et al., 1997). These proteins are found in mitochondria, where they associate into multimeric membrane complexes that degrade unassembled subunits of inner membrane components, including cytochrome oxidase and the ATP synthase. In animals, a handful of ESTs with similarity to FtsH have been found in humans and *C. elegans* (Beyer , 1997), but these have not been characterized. Of the *FtsH*-like genes in plants, only two have been studied to an appreciable extent, viz., the Arabidopsis *FtsH*1 homolog (Lindahl et al., 1996) and *Pftf* from red pepper (Hugueney et al., 1995). The Arabidopsis protein is localized on stromally-exposed regions of the thylakoid

membrane, and its expression is light-regulated (Lindahl et al., 1996). Both of these characteristics are similar to VAR2.

Pftf, which bears the highest similarity to VAR2 of any FtsH homolog, was isolated as a soluble factor that promotes membrane fusion and/or translocation events in an *in vitro* vesicle fusion assay using chromoplast membrane vesicles from red pepper fruit (Hugueney et al., 1995). The gene was subsequently isolated from an expression library. The activity of Pftf in the vesicle assay requires ATP, and Pftf mRNAs are expressed in leaves and developing fruits. Because Pftf was isolated as a soluble factor, it was assumed to reside in the stroma. However, this location is puzzling since the Pftf sequence contains the two transmembrane domains that are a hallmark of FtsH proteins. Nevertheless, the high sequence similarity of VAR2 and Pftf, as well as the similarity in their expression patterns, suggest that the two may have similar activities.

VAR2 functions in thylakoid membrane biogenesis

To our knowledge, VAR2 is the first *FtsH* mutant reported in a multicellular organism. It is also one of the few mutants in higher eukaryotes in the broader class of AAA proteins (Beyer, 1997). Our analyses support the idea that VAR2 functions in thylakoid membrane biogenesis. This is based on our assumption that the various plastid types in heteroplastidic cells of the yellow and white *var2* sectors are intermediates in chloroplast biogenesis, rather than differing products of plastid photooxidation. Photooxidized plastids arise from a lack of colored carotenoids, which normally quench triplet chlorophyll and prevent the generation of reactive oxygen species (reviewed in Demmig-Adams et al., 1996). In carotenoid-deficient plants, the extent of photooxidative damage is proportional to the

light intensity. For instance, in the *immutans* variegation mutant of *A. thaliana*, which is defective in the PDS step of carotenogenesis, the extent of white sector formation is directly proportional to growth illumination: nearly all-green plants develop at low light intensities, variegated plants at moderate light intensities, and albino seedlings at high light intensities (Wetzel et al., 1994). Sector formation in *var2*, on the other hand, is only influenced by light when seedlings are grown at very low intensities (<15 uE) (Chen et al., 1999). Under these conditions, *var2* plants grow slowly, are small at maturity, and are nearly all-green. Their morphology resembles that of wild type plants grown under the same conditions. We conclude that the differing plastid morphologies in *var2* most likely represent plastids in various stages of development.

The hypothesis that VAR2 functions in thylakoid biogenesis is consistent with our finding that the expression of VAR2 in *E. coli* gives rise to an *fts* phenotype (Ogura et al., 1991). Although the molecular details of *fts* are not understood, one possibility is that VAR2 acts in a dominant negative fashion to poison endogenous FtsH interactions that are required for membrane fission/fusion events. An involvement of VAR2 in membrane fusion events would also be consistent with the studies showing a requirement for Pftf activity in chromoplast vesicle fusion *in vitro* (Hugueney et al., 1995). In addition to these activities, VAR2 may be involved in other membrane modeling events during thylakoid biogenesis. Several proteins that serve as substrates of *E. coli* FtsH are found in plastids, such as SecY and F_oa (Merchant, 1985; Roy and Barkan, 1998), and thus it is possible that VAR2 promotes thylakoid development by degrading unassembled forms of these proteins. In support of this notion, a plastid FtsH has been implicated in the degradation of unassembled Rieske FeS protein (RISP) in the thylakoid membrane (Ostersetzer and Adam, 1997). Whether this

activity is due to VAR2 and/or to another plastid-localized FtsH-like protein is not known. Also of relevance in this context is our observation that the quantum yield of PSII (on a per chlorophyll basis) is lower than normal in the yellow and white sectors of *var2* (M. Chen, L. Daley, G. Edwards and S. Rodermel, unpublished). This suggests that pigment-containing plastids in the heteroplastidic cells, though they resemble normal chloroplasts, are not fully functional. VAR2 may thus be involved in promoting normal PSII function, perhaps by regulating the accumulation or integration of thylakoid components.

Figure 7 presents a working model of VAR2 function. In our model, VAR2 is an element that functions in thylakoid membrane biogenesis after proplastids have increased in size and become partially differentiated. Our rationale for this is that plastids in heteroplastidic cells of the yellow and white sectors, regardless of their morphology, are the size of normal chloroplasts, versus much smaller proplastids. We assume that VAR2 is involved in some event(s) involving vesicle formation from the inner envelope, vesicle fusion, and/or modeling of the thylakoid membrane. In the absence of this activity, large vesicles accumulate in the stroma. VAR2 does not appear to be a general plastid membrane biogenesis factor inasmuch as etioplasts have a normal morphology. Rather, VAR2 function may be restricted to the biogenesis of thylakoids. Consistent with this interpretation, VAR2 is expressed only in green organs of the plant and its expression is light-regulated, as is true of most genes whose products are involved in photosynthesis (Mullet, 1988; Leon et al., 1998). The latter finding further implies that VAR2 is involved in the conversion of etioplasts to chloroplasts. Barnes et al. (1996) have shown that plastids with a vacuolated morphology similar to those in *var2* are intermediates in this etioplast-to-chloroplast

differentiation process. However, we do not know whether VAR2 acts before and/or after this stage.

Mechanism of *var2* variegation

In designing a model of the mechanism of *var*2 variegation, we start with the assumption that green, normal-appearing tissues arise as a consequence of either partial VAR2 function or a compensating activity. We favor the latter hypothesis because the alleles we have sequenced are predicted to have mutations in key sites, including the putative ATP-binding site (*var*2-3 and *var*2-5). *var*2-2 has an altered amino acid immediately adjacent to the predicted end of the second transmembrane domain. While it is not obvious how this mutation affects function, one possibility is that it affects integration of the protein into the membrane, or perhaps its targeting to the membrane, as observed for mutations immediately adjacent to transmembrane segments of Golgi apparatus proteins (Munro, 1998). On the other hand, the *var*2-1 allele has a nonsense codon in its C-terminus and is predicted to code for a truncated protein. *var*2-1 and *var*2-2 have much reduced mRNA amounts, and VAR2-1 and VAR2-2 proteins cannot be detected on Western immunoblots (Y. Choi, M. Chen and S. Rodermel, unpublished data). It is therefore likely that *var*2-1 and *var*2-2 approximate null phenotypes. The presence of normal green tissue in these mutants suggests that there must be an activity that is able to compensate for the lack of VAR2, at least in some cells.

Proceeding from the notion that there is a compensating VAR2 activity, there are two possibilities. One is that there is a redundant VAR2 function, i.e., mediated by another protein. A second possibility is that the events catalyzed by VAR2 can occur at a low rate

even in its absence. Regardless of which (if either) of these is correct, we hypothesize that plastids with a threshold of this compensating activity are able to differentiate into green, fully functional plastids. Once attained, this state is capable of being propagated, giving rise to a clone of green plastids and cells. Plastids with progressively less of this compensating activity have decreased plastid function, and white plastids are devoid of this activity. Clones of white plastids (and cells) are capable of arising because white plastids can divide as rapidly as green ones (Kirk and Tilney-Bassett, 1978), requiring only imported nuclear gene products for their division.

To explain the tissue-wide patterns of variegation, we hypothesize that the accumulation of the compensating activity depends on an interplay of factors that include cell-specific expression and local differences in rates of cell and plastid division. Similar factors are generally known to contribute to the specification of developmental patterns in plants and animals. In support of this hypothesis, nearly all-green *var2* plants are produced under conditions that retard growth (e.g., dim light) (Chen et al., 1999), suggesting that decreased rates of cell and plastid division might allow accumulation of the compensating activity to a threshold in a larger proportion of cells. Consistent with this notion, cotyledons normally express high levels of VAR2, but they appear normal (all-green) in all of the *var2* alleles we have examined. Because cotyledon cell division is completed in the embryo prior to seed formation (Bowman, 1994), it is possible that cotyledons are green because the compensating VAR2 activity has time to accumulate to threshold levels in the absence of cell division prior to germination.

According to our working model, the stochastic pattern of variegation in the expanding *var2* leaf reflects, to a large extent, the pattern established in the leaf primordium,

since this is where most cell divisions occur. This pattern would become manifest during expansion as the gradual emergence of green islands against a yellow background. We have previously suggested that the yellow color is an optical illusion caused by heteroplastidic cells, i.e., some chlorophyll-containing plastids against a background of white plastids, because pigment precursors do not accumulate in the mutant (Chen et al., 1999). As expansion proceeds, plastid divisions would continue to occur and the leaf would become more green, perhaps because of gradual accumulation of the compensating activity. Presumably, the compensating activity would not be able to rescue a white plastid sometime after full expansion is attained, since white sectors do not become green after this stage. The attainment of full expansion usually marks the beginning of the senescence phase of leaf development, when rates of photosynthesis begin to decline, concomitant with reduced rates of synthesis and increased turnover of chloroplast components (reviewed in Bleecker and Patterson, 1997).

We have recently proposed that a compensating activity is responsible for the generation of normal green tissues in *immutans*, for which we have yet to isolate a non-null allele (Wu et al., 1999). Based on our current working model of *var2* variegation, we suggest that compensating activities may be a ubiquitous mechanism to explain variegations produced by lesions in nuclear or plastid genes. Such variegations may comprise the majority of variegations in nature (Tilney-Bassett, 1975; Steeves and Sussex, 1989). Compensating activities may also be responsible for the phenotypes of various non-variegated pigment mutants (e.g., Jarvis et al., 1998). We do not know the nature of the compensating activity in *var2*, but one possibility is that it is another FtsH-like protein. We are currently testing this hypothesis.

METHODS

Plant material

Five *var2* alleles have been isolated (Martínez-Zapater, 1992; Chen et al., 1999). These alleles were generated by X-ray or EMS mutagenesis, and all are in the Columbia (Col-0) background. For positional cloning of *var2*, an F2 mapping population was generated by crossing the *var2-4* ("yellow variegated") allele with Landsberg *erecta*. A total of 2080 chromosomes (1040 F2 plants) were analyzed to generate the recombinant pool.

DNA manipulations

The Arabidopsis Biological Resource Center (ABRC, Ohio State University) provided several libraries for use in the positional cloning experiments: four yeast artificial chromosome (YAC) libraries (Ecker, 1990; Ward and Jen, 1990; Grill and Somerville, 1991; Creusot et al., 1995), the TAMU bacterial artificial chromosome (BAC) library (Choi et al., 1995), the pOCA18 cosmid library (Schulz et al., 1994), and the _PRL2 cDNA library (Newman et al., 1994). References that describe these libraries include protocols for library maintenance and screening and for the isolation of cloned DNAs. Pulsed-field gel electrophoresis was used to estimate the sizes of YAC and BAC clones (Schmidt et al., 1996).

Cleaved amplified polymorphic sequence (CAPS) markers (Konieczny and Ausubel, 1993) were used in mapping *var2*. These markers were generated to the ends of YAC, BAC and cosmid inserts, as well as to Arabidopsis DNAs cloned into pBluescript SK- (Stratagene, LaJolla, CA). Plasmid rescue was used to isolate YAC left ends (sequences adjacent to the

bacterial origin of replication and selectable marker) (Schmidt et al., 1996), and *pyr*F complementation was used to isolate YAC right ends (Wright et al., 1997). BAC and cosmid end clones were isolated by inverse polymerase chain reaction (iPCR) or by direct subcloning. PCR, iPCR, and long PCR were performed by established methods (Ausubel et al., 1998). Other polymorphic sequences used in the chromosome walk included nga361, a simple sequence length polymorphism (SSLP) marker (Bell and Ecker, 1994), CAPS marker m283 (ABRC) and a CAPS marker generated to COP1 (gift of Robin Buell, Carnegie Institution of Washington).

Procedures for DNA gel blot and colony hybridizations and for the isolation of Arabidopsis DNA have been described (Wetzel et al. 1994; Wu et al., 1999). DNA sequencing was performed by the Iowa State University Nucleic Acids Facility, and DNA sequences were analyzed using programs within the Genetics Computer Group (Madison, WI) software package (Devereux et al., 1984). Phylogenetic analyses were conducted by the neighbor-joining algorithm using Phylip (Saitou and Nei, 1987; Felsenstein, 1993). The wild type *VAR*2 cDNA sequence has been submitted to the DDBJ/EMBL/GenBank databases as accession number AF135189.

RNA manipulations

Procedures for the isolation of RNA are described in Wetzel and Rodermel (1998). RNA gel blot analyses were conducted as described in Wetzel et al. (1994). The blots were probed with an insert from a *VAR*2 cDNA clone (p_ZLVAR2). The band intensities were quantified by phosphorimage analysis.

Chloroplast import assays

The import of radiolabeled VAR2 into isolated pea chloroplasts was analyzed by established procedures (Cline et al., 1989; Perry et al., 1991). Briefly, a full-length *VAR2* cDNA (p_ZLVAR2) was transcribed and translated in a coupled system (Promega) using ^{35}S-methionine. The import mixture contained intact chloroplasts from 8- to 10- week old pea seedlings (1 mg/ml of chlorophyll), 10 mM MgATP, 0.33 M sorbitol, 50 mM HEPES (pH 8.0), 10 mM methionine and the labeled VAR2 protein. Following a 30 min incubation at 25°C, the chloroplasts were treated with thermolysin (0.2mg/ml, 4°C, 30 min) to remove labeled proteins adsorbed to the outer surface of the organelles. The plastids were then lysed by incubation in 25 mM HEPES (pH 8.0), layered onto a sucrose step gradient (0.46 M, 1 M, and 1.2 M sucrose), and centrifuged for 1 hour in a Beckman SW55.1 rotor (48,000 rpm). The top layer (0.5 ml) contains the stromal fraction, and stromal proteins were isolated by precipitation with an equal volume of acetone; the pellet was resuspended in 100 ul of SDS-PAGE running buffer. Envelope proteins sediment at the 0.46 M/1 M gradient interface, and they were isolated by ultracentrifugation of 0.5 ml of this fraction (Beckman SW55.1 rotor, 48,000 rpm, 1 hour); the pellet was resuspended in 100 ul of SDS-PAGE running buffer. Finally, the thylakoids are found in the pellet, and they were resuspended in 100 ul of import buffer, then divided into three equal aliquots. One aliquot was washed with an equal volume of 0.2 N NaOH; the second was incubated with 0.3 mg/ml of trypsin (0°C, 30 min); and the third received no further treatment. The membranes were collected by centrifugation (as above), washed in import buffer, and resuspended in 30 ul each of SDS-PAGE running buffer. Equal volumes (30 ul) of the stromal, envelope, and of each the three thylakoid

samples were electrophoresed through 12% SDS polyacrylamide gels. After electrophoresis, the gels were fluorographed as described by Perry et al. (1991).

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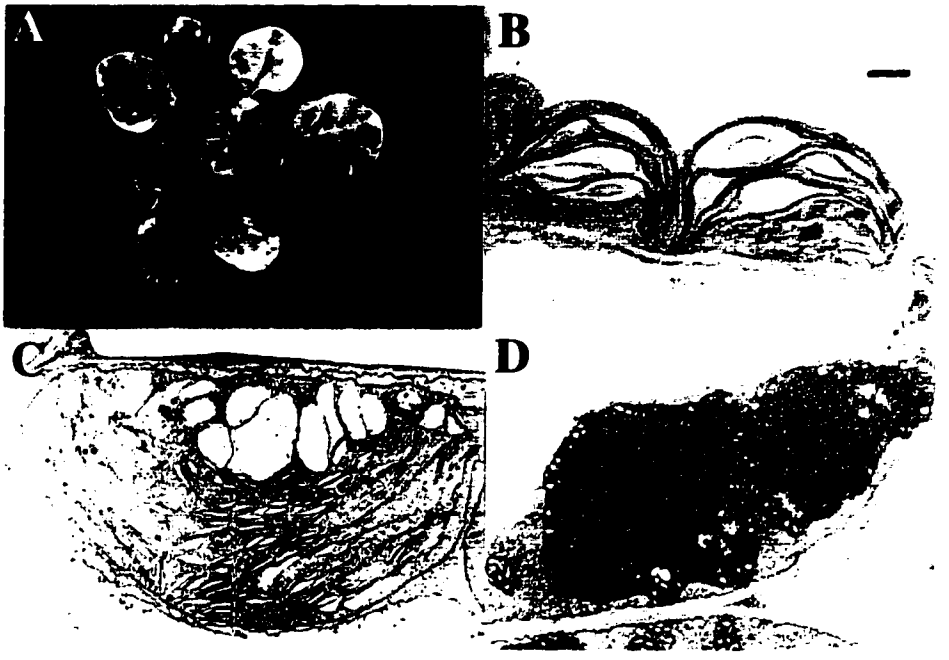


Figure 1. Morphology and ultrastructure of *var2*.
A) 10 day old light-grown seedling. Electron micrographs of typical plastids from a green sector (B), from a heteroplastidic cell of a yellow sector (C), and from an etiolated, 10 day old dark-grown seedling (D). Bar is 1 μm.

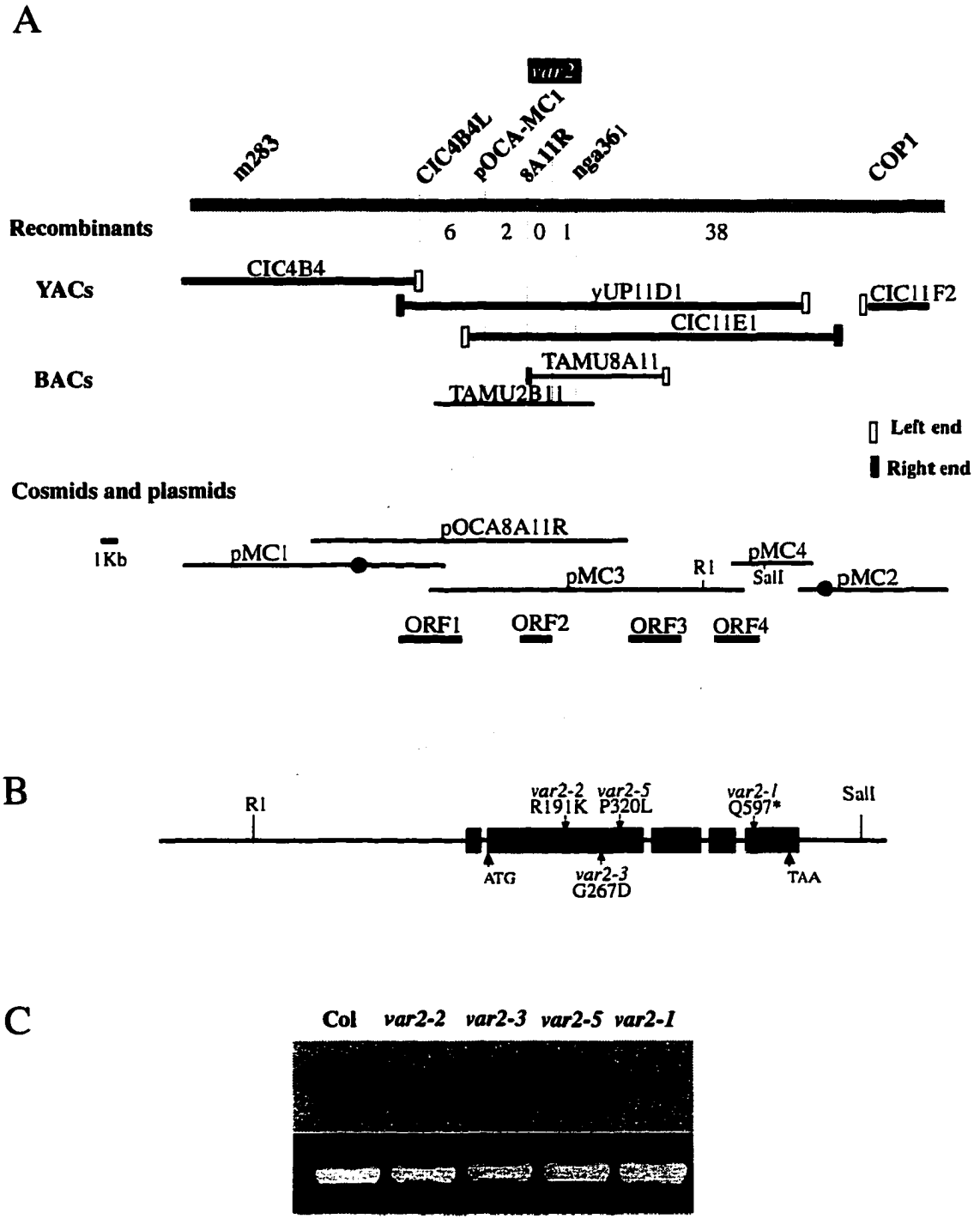


Figure 2. Positional cloning and identification of *VAR2*.
A. Chromosome walk to *var2*.
B. Structure of *VAR2* and mutations in *var2* alleles.
C. mRNA expression in *var2* alleles.

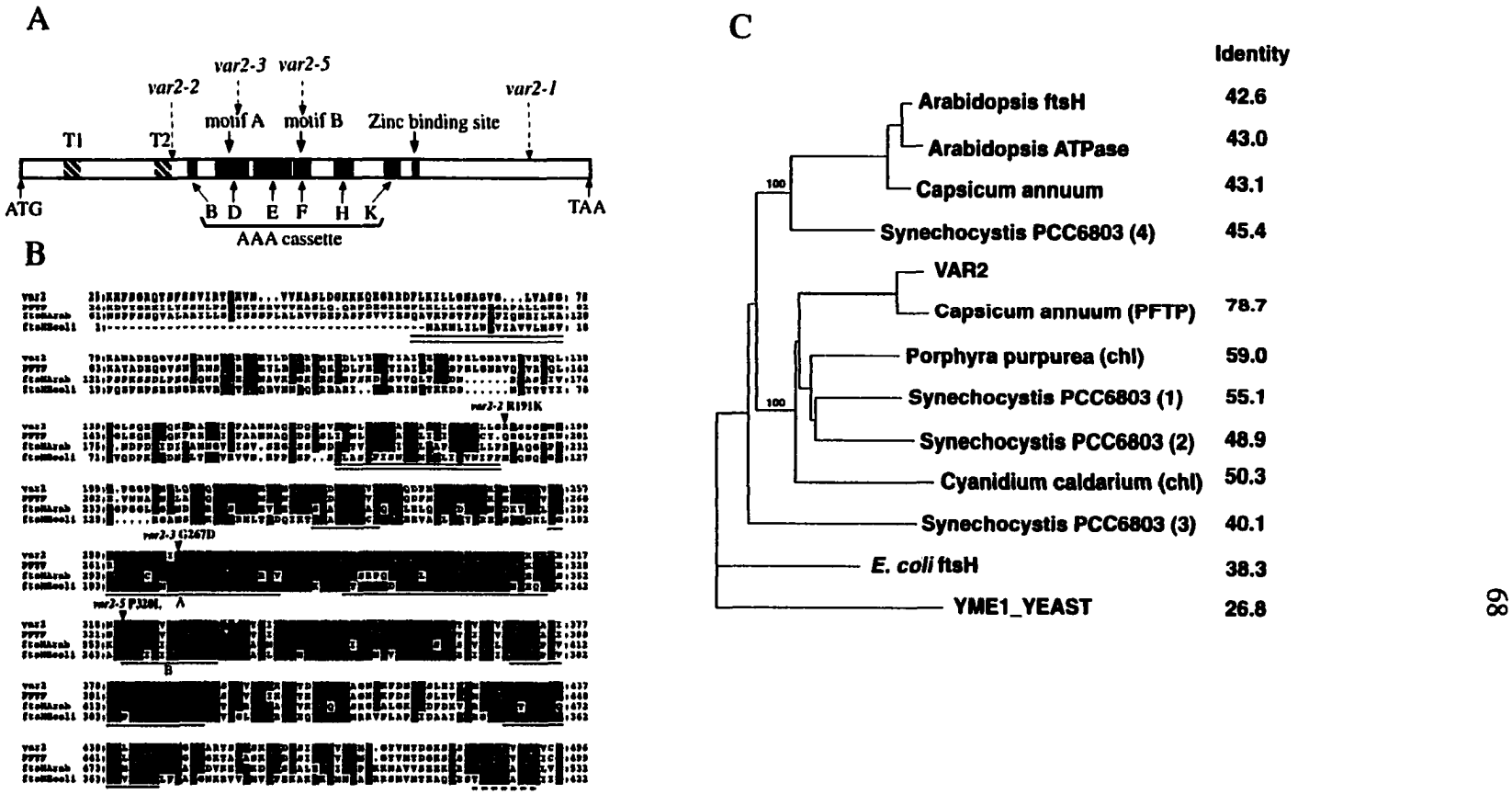


Figure 3. VAR2 is an FtsH homolog.
A. Schematic of conserved domains in *VAR2* cDNA. VAR2 has a single AAA cassette with six domains (B, D, E, F, H and K).
B. Comparison of VAR2 with FtsH from diverse organisms. Transmembrane helices are double-underlined. Conserved domains of the AAA cassette are single-underlined;
Walker boxes A and B are shown. The zinc-binding domain is indicated by dashes. Amino acid identities are shaded. Mutations in the various alleles are indicated above the sequence.
C. Phylogenetic analysis of FtsH-like proteins from photosynthetic organisms. Relationships were determined by the neighbor-joining distance method using Phylip (Saitou and Nei, 1987; Felsenstein, 1993). Bootstrap values (100 replicates) are shown for the two major branches. Amino acid identity with respect to VAR2 are shown. Codons 25-496 of the VAR2 sequence were used for comparison (as in Figure 3B).

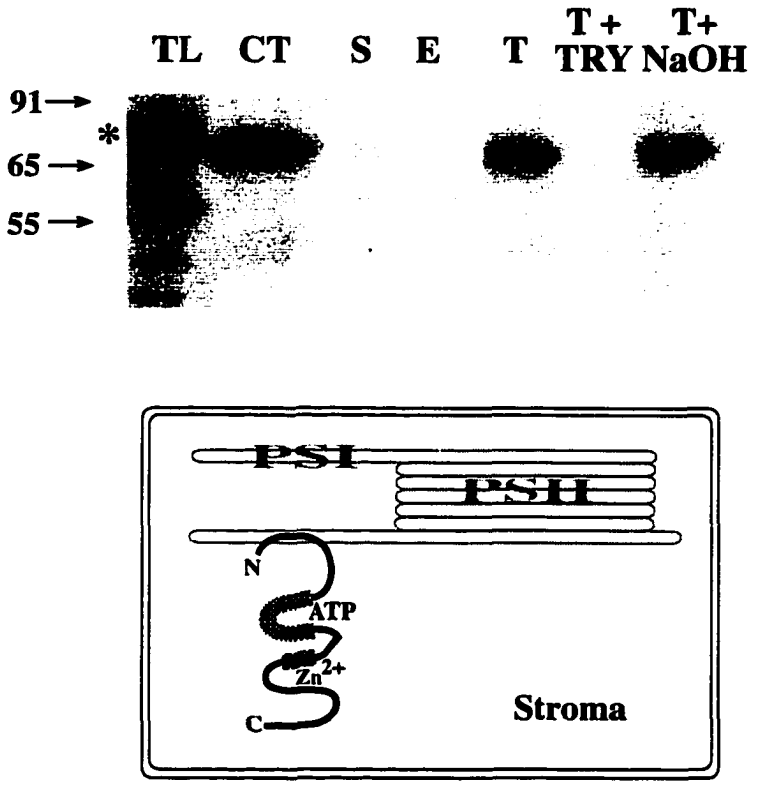


Figure 4. Chloroplast import assays.
A. VAR2 was transcribed and translated in vitro with ^{35}S methionine and incubated with isolated pea chloroplasts. After import, the chloroplasts were treated with thermolysin. The "TL" lane is an aliquot of the transcription/translation mix prior to import, and the "CT" lane contains 1/10 of the total reaction mixture after thermolysin treatment. After the organelles were lysed and fractionated on a step sucrose gradient, the stromal ("S"), envelope ("E") and thylakoid ("T") fractions were collected. Aliquots of the thylakoid fraction were treated with trypsin ("TRY") or NaOH. Equal volumes of each fraction were electrophoresed through a 12% SDS-polyacrylamide gel, and the labeled protein bands were detected by fluorography. *, the position of the precursor VAR2 protein in the transcription translation mix. B. Schematic of the topography of VAR2 in the thylakoid membrane. The C-terminus of VAR2 is exposed to the stroma. Stroma lamellae are enriched in PSI and grana stacks are enriched in PSII (Green and Durnford, 1996).

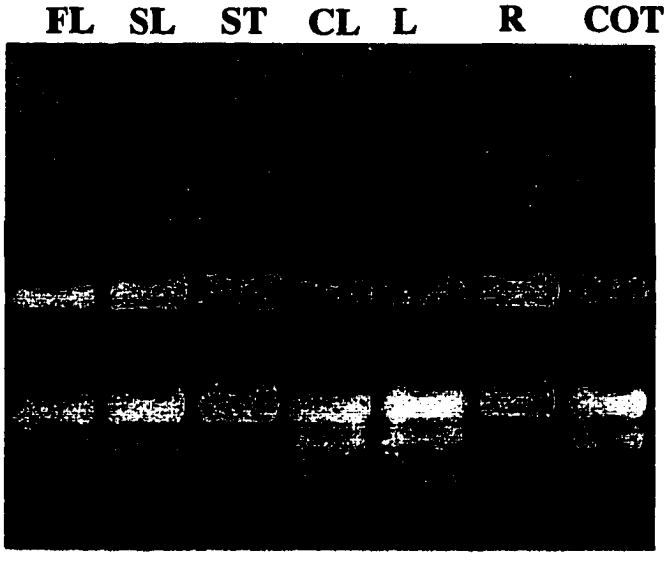


Figure 5. *VAR2* mRNA expression.
RNA gel blot analyses were conducted as described in the legend to Figure 2C using total cell RNAs from A) Arabidopsis organs and B) 11 day-old etiolated Arabidopsis seedlings ("DK") exposed to light for 3, 6 or 12 hours. 11 day-old light-grown seedlings ("LT") served as controls. "FL", whole flowers, "SL", siliques, "ST", stems, "CL", cauline leaves, "L", mature leaves; "R", roots, "COT", cotyledons.

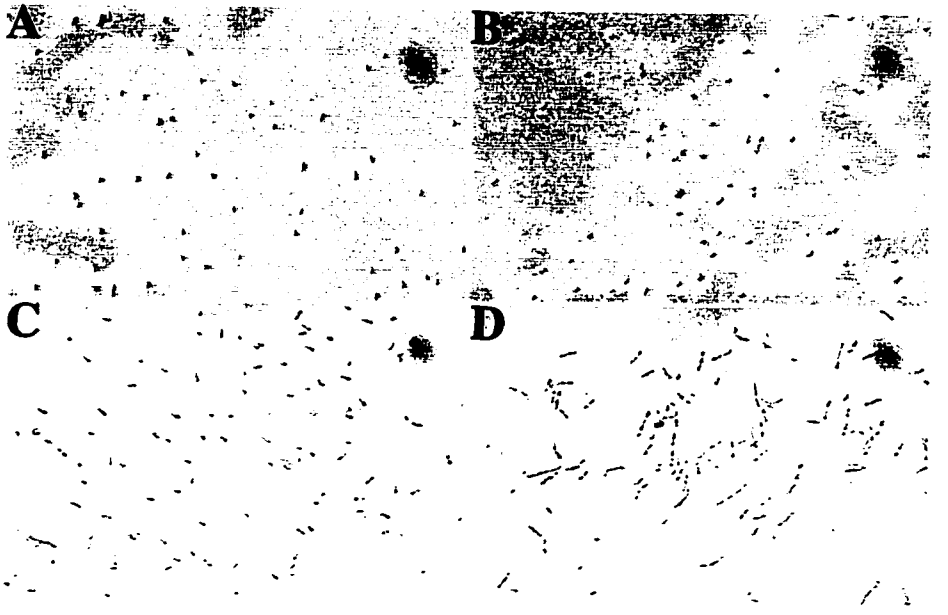


Figure 6. *VAR2* expression in *E. coli* gives rise to an *fts* phenotype.
E. coli were transformed with a full-length VAR2 cDNA cloned into pBluescript (p_ZLVAR2). The cells were grown in M9 medium and *VAR2* expression was induced by the addition of IPTG. At induction, cells were transferred to 30°C (C) or 42°C (D) for 5 hours. pBluescript expressing B-galactosidase served as controls at 30°C (A) and 42°C (B).

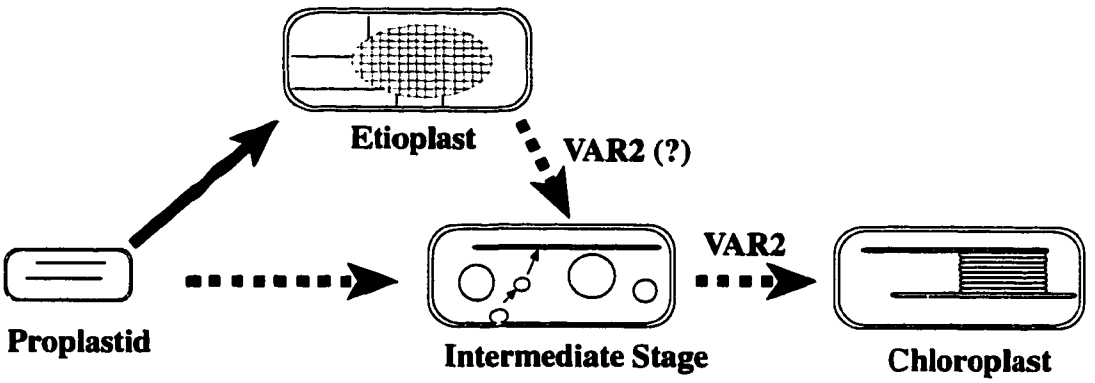


Figure 7. Proposed function of VAR2 in plastid membrane biogenesis. Proplastids are converted to chloroplasts in light-grown seedlings and to etioplasts in dark-grown seedlings. We propose that the vacuolated plastids and plastids with rudimentary lamellar structures in *var*2 are intermediates in the process of thylakoid membrane biogenesis, and that VAR2 is involved in thylakoid formation by mediating membrane fission/fusion events involving the inner envelope membrane. Upon illumination, the prolamellar bodies in etioplasts disperse and form thylakoids. This process proceeds via an intermediate (Barnes et al., 1996) that resembles the vacuolated plastids in *var*2. VAR2 may act either in the formation of this intermediate or to convert this intermediate to mature thylakoids.

CHAPTER 4. GENETIC AND BIOCHEMICAL CHARACTERIZATION OF VAR2

INTRODUCTION

The *var2* locus in Arabidopsis encodes a FtsH-like zinc-dependent protease, which belongs to an ancient large gene family called AAA-ATPase (ATPase associated with a variety of cellular activities) (Chen et al., 1999). All members of this gene family contain one or two copies of an approximately 250 amino acid ATP binding domain called the AAA cassette (see review, Beyer 1997; Neuwald et al., 1999; Patel and Latterich 1998; Swaffield and Purugganan 1997). The mechanism of how the AAA cassette functions is poorly understood. As discussed in chapter 3, AAA-ATPase proteins can be can be divided into subfamilies based on the number of AAA cassettes and other distinctive functional domains (Beyer 1997). VAR2 belongs to a subfamily that has a zinc binding site, HEXXH, for metallopeptidase. Some members in this subfamily have been studied, including *E. coli* FtsH (Granger et al., 1998; Schumann 1999; Tomoyasu et al., 1993; Tomoyasu et al., 1993), PFTF (Protein Fusion and Translocation Factor) from red pepper (Hugueney et al., 1995), YME (Yeast Mitochondrial Escape) (also called YTA11) (Thorsness et al., 1993), YTA10 (Tauer et al., 1994), and RCA (Respiratory Chain Assembly) (Tzagoloff et al., 1994) (also called YTA12) from yeast (Paul and Tzagoloff 1995; Schnall et al., 1994). The structure and function properties of these proteins have provided clues about how VAR2 might be involved in thylakoid membrane biogenesis.

First, structurally most of these ATP dependent metallopeptidases form either homo- or hetero- oligomers as a functional unit (Neuwald et al., 1999). In *E. coli*, the functional

unit of FtsH is a homo-oligomeric complex (Akiyama et al., 1994; Akiyama et al., 1994). The periplasmic region (i.e. the fragment between two transmembrane segments) of FtsH is responsible for binding to other FtsH proteins, whereas the cytoplasmic domains are required for the function of FtsH. This notion is clearly demonstrated by the phenotypes of *ftsH* mutants. When wild type *E. coli* cells carry a plasmid with a mutant copy of FtsH, the mutation can give rise to either dominant or recessive phenotypes. Mutations in the periplasmic domains lead to a recessive phenotype, whereas point mutations at the C-terminus are dominant negative, because the mutant protein can still form oligomers with the native wild type FtsH and poison the complex (Akiyama et al., 1994). Besides forming homo-oligomers, FtsH also tightly associates with two other proteins called HflK and HflC. HflK/C serve as modulators for FtsH peptidase activity towards different selective substrate proteins (Kihara et al., 1996). In the case of yeast YTA10 and YTA12, each of these highly similar (53% identity) ATP dependent metallopeptidases forms an approximately 250kDa homo-oligomer. These two homo-multimeric oligomers assemble to make an 850kDa protein complex. The formation of this bigger complex is nucleotide dependent (Arlt et al., 1996).

Secondly, in terms of function, some of the proteins in this subfamily have selective peptidase activity and degrade unassembled proteins. For FtsH in *E. coli*, several substrates have been determined, including membrane proteins, such as unassembled SecY (one of the subunits of the protein translocation complex) (Akiyama et al., 1996) and the *a* subunit of the F_0 complex of the H^+-ATPase (Akiyama et al., 1996), and soluble proteins, such as sigma 32 (heat shock sigma factor) (Herman et al., 1995) and phage lambda CII (transcriptional activator) (Kihara et al., 1997). In the case of the YTA proteins in yeast, YTA11 (YME) is

involved in the degradation of subunit 2 of the cytochrome c oxidase in yeast mitochondria (Nakai et al., 1995). YTA10 and YTA 12 (RCA) mediate the degradation of some unassembled subunits of the respiratory chain complexes (Tzagoloff et al., 1994).

Due to the clear presence of the zinc binding site for metallopeptidase, determination of substrates and confactors were the main focus of early research on these ATP-dependent metallopeptidases. However, more recent evidence has shown that there might be another equally, if not more, important function of these proteins. This is a chaperone-like activity. It was first shown in *E. coli* that FtsH is required for integration of membrane proteins, and that this does not involve proteolysis. Also, the ftsH phenotype could be partially rescued by over-expressing chaperones (Shirai et al., 1996). The YTA10 and YTA12 complex is similarly required for assembly of the membrane-associated ATP synthase and the respiratory chain complexes in yeast (Paul and Tzagoloff 1995; Tzagoloff et al., 1994). A recent paper further illustrated that the AAA cassette in YTA11 (YME) can bind to unfolded polypeptides and prevent them from aggregation (Leonhard et al., 1999).

The question arises why two rather opposite functions (i.e., chaperone and protease) are present in the same protein. In the past two years, three review papers have unified these two functions by suggesting that the ATP dependent metallopeptidases serve in protein quality control (Gottesman et al., 1997; Suzuki et al., 1997; Neuwald et al., 1999). On one hand, the chaperone functions help protein complex assembly or dissociation; on the other hand, the metallopeptidase activities degrade any mis-assembled or dissociated components.

In this chapter, I used molecular and biochemical methods to try to address the structural and functional properties of VAR2. First, more *var2*-like mutants were obtained and examined to determine if they were *var2* alleles. Second, gel filtration and

coimmunoprecitation were employed to characterize the VAR2 protein complex. Third, a VAR2-GFP fusion construct was made to localize VAR2 *in vivo*. Fourth, a 35S promoter-driven antisense FtsH1 was constructed to examine whether FtsH1 is the redundant function for VAR2. Fifth, I tried to develop an assay to monitor the protease activity of VAR2. I hope the experiments and ideas discussed in the chapter will serve as either preliminary results or the basis for ideas for future studies.

MATERIAL AND METHODS

Sequencing *var2* alleles

To sequence either the genomic *VAR2* DNA or the RT-PCR product amplified from *var2* mRNA, primers were designed based on the cDNA genomic sequences of *VAR2*. The names and positions of these primers are presented in Figure 1. The 5' to 3' sequences of the primers are: F2: gttctgtctcatcgttcatg; R2: cagaaccagagatggagaag; F3: ctgcggttggtgccaagatc; R3: tgatatcgatgtccgtgctc; F4: gagcagatctcgctaacctc; R4: ctgcaagcttctcagacatg; F5: agttatgctggagcctgatg; R5: tggtgttctgcttgtgctac. Primers with the same number were used as a

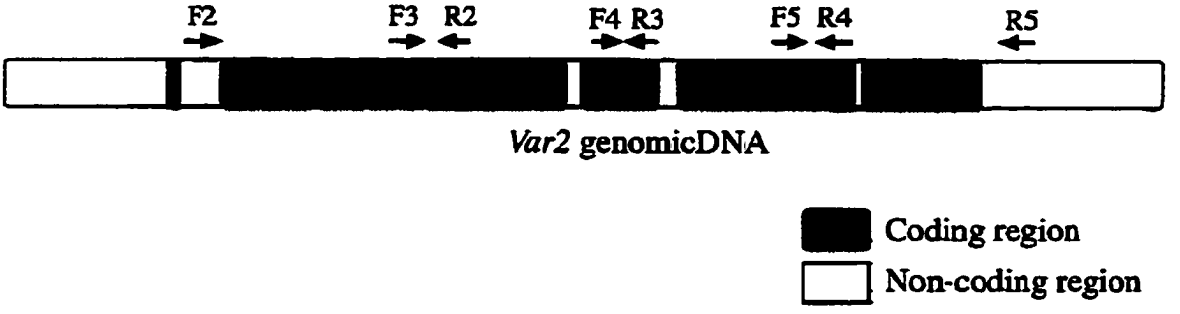


Figure 1. Positions of the primers used to sequence the genomic DNA of *var2* alleles.

pair. Therefore, 4 PCR reactions can cover the whole genomic sequence of the coding region.

Gel filtration

Pea chloroplasts were isolated as described in chapter 3. A detailed gel filtration protocol is described in the *Current Protocols in Molecular Biology* (Ausubel et al., 1997). In brief, chloroplasts were resuspended in Buffer A (0.4mM sucrose, 10mM NaCl, 50mM HEPES pH 7.0, 2mM Na-EDTA, 1mM PMSF, 4mM $MgCl_2$) containing 5mM ATP. Dodecyl-β-D-maltoside was added to a final concentration of 10 mg/ml and incubated on ice for 15 minutes to solubilize the membranes. The sample was centrifuged at 14,000g for 15 minutes to remove insoluble material. The supernatant was loaded onto a Bio-RAD Macro-Prep 1000/40 column equilibrated with Buffer A plus 5mM ATP and 1% Triton X-100. Proteins in each fraction were precipitated with 10% trichloroacetic acid (TCA), run on 12% PAGE gel electrophoresis, and immunoblotted using VAR2 antibody. Gel filtration standards from Bio-RAD were applied to the column under the same operation conditions to estimate the molecular weight of proteins in each fraction.

Coimmunoprecipitation

Coimmunoprecipitation was done according to a protocol from the *Current Protocols in Molecular Biology* (Ausubel et al., 1997). In brief, pea chloroplasts were isolated and resuspended in either Buffer A or Buffer A with 5mM ATP and incubated for 30 minutes. A final concentration of 10 mg/ml of Dodecyl-β-D-maltoside was used to solubilize the membranes. Insoluble material was removed by centrifugation. The supernatant was first

incubated with protein-A agarose at 4°C overnight. The protein A-agarose was then removed by centrifugation. The solution was incubated with VAR2 anti-serum for 2 hours, followed by the addition of protein A-agarose and 2 hours of incubation at 4°C. The immuno-complex was isolated by centrifugation and washed by 0.1% Triton X-100 in TSA solution with or without ATP. VAR2 protein complex was dissociated from protein A agarose by boiling using SDS-PAGE loading buffer for 10 minutes. The proteins were separated on 12% SDS-PAGE and visualized using silver stain.

RESULTS

Sequence analyses of additional *var2* alleles

One way to understand structure and function relationships of VAR2 is to characterize different *var2* alleles. In order to extend our *var2* allele collection, I ordered all *var2*-like mutant lines from the Arabidopsis stock center at Ohio State University. Based on their phenotypic similarities to *var2* (i.e. normal cotyledons and variegated true leaves), eight of them were chosen for further molecular and genetic characterization. These eight mutant lines are CS3166, CS3279, CS3622, CS3640, CS3647, CS3654, CS3659, and CS3681. Northern blot analysis using total RNAs extracted from plants of each mutant line showed that CS3166 had much less *VAR2* message, and both CS3622 and CS3654 had slightly larger *VAR2* mRNAs than normal. *VAR2* mRNA profiles were unaltered in the other mutant lines (Figure 2). From the Northern analysis on previouse *var2* alleles, we know that the changes in *VAR2* mRNA message indicates that there might be mutations in *VAR2* gene. I chose CS3166 and CS3622 for further analysis to prove that they had mutations in the *var2* locus.

For these analyses, I sequenced the genomic and RT-PCR products of CS3166 and CS3622. In CS3166, I found a G to A at the 3' end of the third intron in the genomic sequence, which disrupted the conserved 3' splicing site. The sequence of the RT-PCR product from this mutant line revealed that the third intron was not sliced out at the original 3' site, but that a cryptic 3' splicing site was used downstream of the original site. The mis-

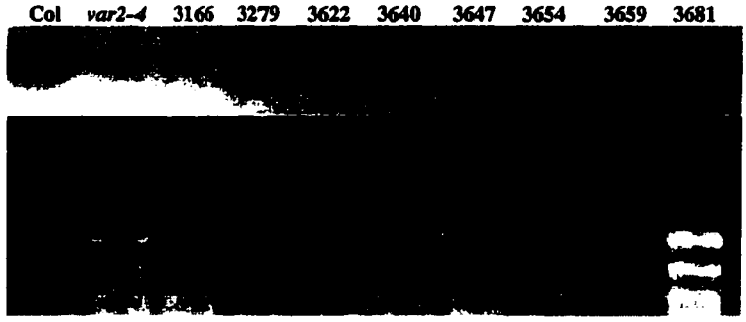


Figure 2. Northern blot on new potential *var2* alleles. The numbers represent the stock number of each mutant line in the Arabidopsis stock center at Ohio State University. The blot was probed by *VAR2* cDNA. The lower panel shows a picture of the original gel, which was used to monitor the loading of the gel.

spliced product caused a frameshift and an early stop codon, which presumably caused an instability of the mutant mRNA. In the case of CS3622, a G to A transition was found at the 3' end of the fourth intron, which altered the conserved intron splicing sequence "AG" to "AA". With such a mutation, the fourth intron was not spliced out in this mutant. Since neither a stop codon nor a frameshift was introduced by the addition of the fourth intron in the mature *var2* mRNA, the result of this mutation led to a slightly bigger but stable mRNA.

VAR2 and green fluorescent protein (GFP) fusion construct

To determine the temporal and spatial expression of VAR2 *in vivo*, I generated a *VAR2* promoter driven *VAR2*/GFP translational fusion. First, an improved version of GFP, pBIN m-gfp5-ER, was obtained from Dr. Kirby Siemering's lab at Cambridge University (Figure 3A). To remove the CaMV 35S promoter, I digested the vector with HindIII and BamHI, polished the ends with T4 DNA polymerase, then rejoined the blunt ends using T4 DNA ligase. The modified vector was named pBIN m-gfp5-ER1. The original GFP protein had an ER targeting sequence at its N-terminus, which was connected to the GFP coding sequence by an EcoRI site as indicated in Figure 3. I inserted a 4.3Kb EcoRI genomic DNA, which contained 2Kb of the *VAR2* promoter region and most of the *VAR2* coding region, into the EcoRI site, generating a fusion protein. Because the ER targeting sequence was separated from the rest of the coding sequence, the VAR2-GFP protein would be targeted to where the native VAR2 localizes. The VAR2-GFP fusion construct was called pBIN V-GFP. pBIN V-GFP was used to transform both wild-type Col-0 and *var2-1* using *Agrobacterium*-mediated transformation. Transformed plants were selected on MS plates containing kanamycin and confirmed by PCR using NPTII specific primers and Southern blots using NPTII probes. The T1 generation of *var2-1* transformed with pBIN V-GFP showed the same phenotypes as *var2-1*, which suggested that the VAR2-GFP fusion protein could not complement the *var2* phenotype. However, T1 generation plants of Col-0 transformed with pBIN V-GFP surprisingly had green cotyledons and yellow young leaves, which resembled the *var2* phenotype. This dominant negative phenotype is consistent with C-terminus *ftsH* mutants of *E. coli*, and suggests that the VAR2-GFP fusion proteins poison the native VAR2 proteins by forming complexes with them. Further phenotypic and

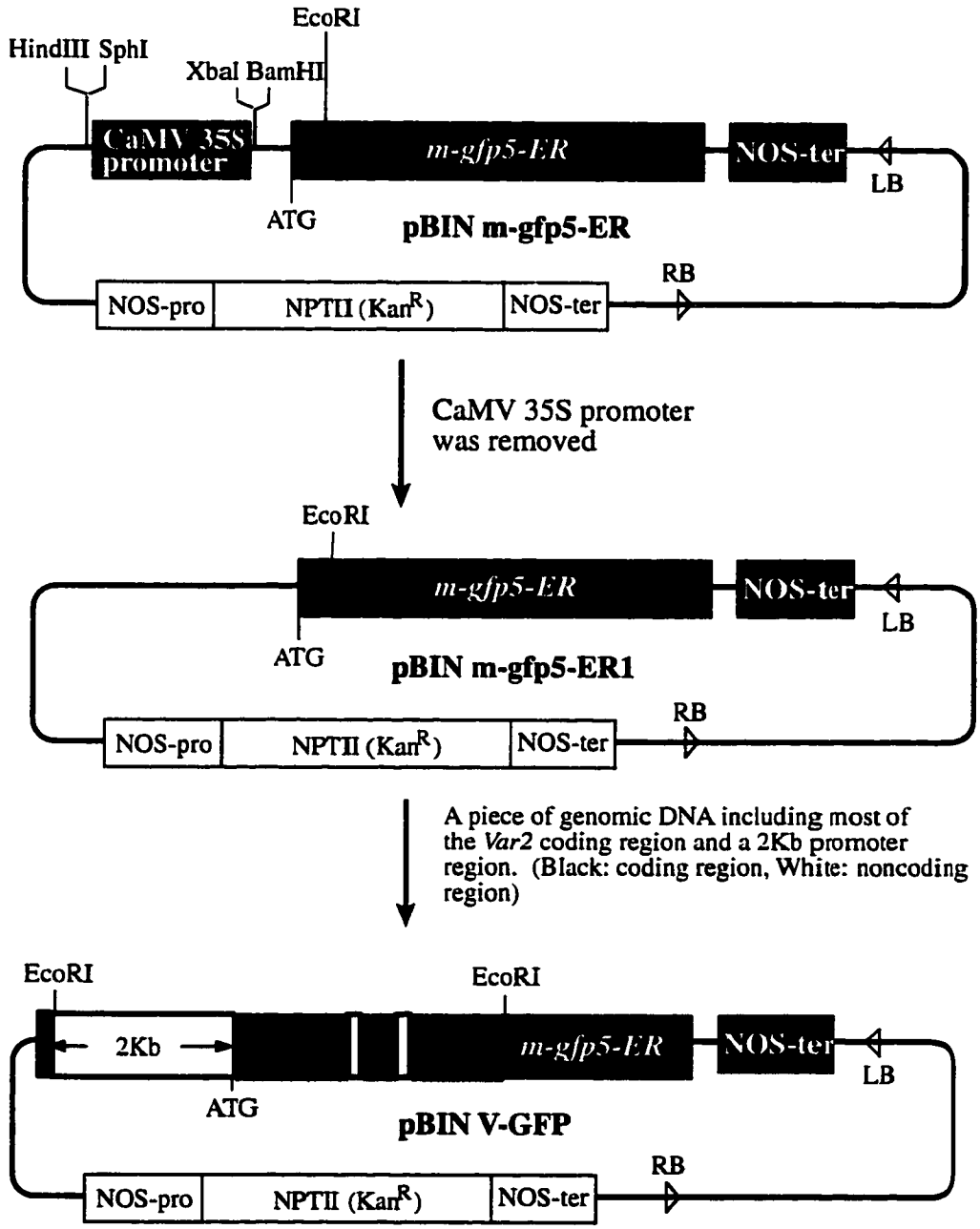


Figure 3. Construction of the VAR2-GFP construct

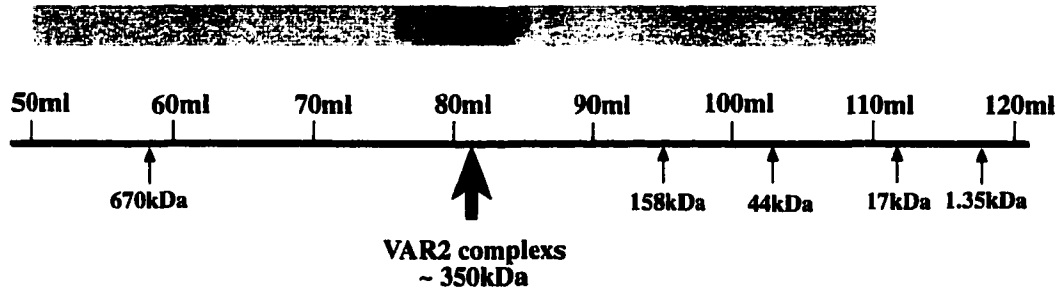
molecular analyses are required on the T2 generation of these transformed plants. The dominant negative phenotype needs to be carefully examined, and the expression level of the VAR2-GFP protein will be determined using Western blot with VAR2 antiserum. Since the VAR2-GFP construct caused the dominant negative phenotype, it indicates that VAR2-GFP fusion protein must locate at the same place as the native VAR2. Therefore, these transfromed plants are still useful for examining VAR2 subcellular localization using GFP as a visible marker.

VAR2 forms a protein complex

The dominant negative phenotype of *E. coli* transformed with *VAR2* and wild type plants transformed with the VAR2-GFP fusion construct suggest that VAR2 might function as an oligomer, similar to FtsH in *E. coli*. To test this hypothesis, I isolated chloroplasts from pea leaves and solubilized the membranes under non-denaturing conditions either with or without ATP. The solubilized proteins were subjected to get filtration chromatography using Macro-Prep 1000/40 (Bio-RAD). The VAR2 protein eluted in fractions 38-40, which corresponds to a molecular weight about 350kDa (Fig 4A). This is much larger than the molecular mass of the VAR2 monomer (69kD).

To further investigate whether the VAR2 protein complex is homomeric or heteromeric, I solubilized the VAR2 complex as described above, coimmunoprecipitated the complex with VAR2 antiserum, separated the protein complex using 12% SDS-PAGE, and visualized the protein bands using silver stain. When the complex was precipitated without ATP, only VAR2 protein was detected. An extra band was precipitated with VAR2, when ATP was present (Fig 4B).

A



B

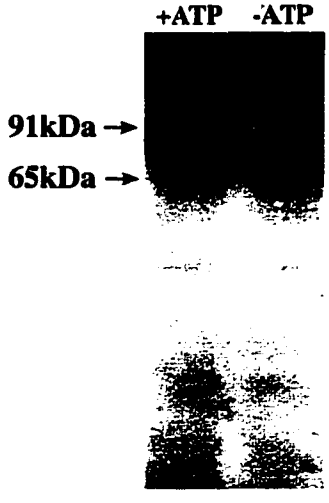


Figure 4. VAR2 proteins form a 350kD complex.
A. Western blots on fractions from gel filtration experiments using VAR2 antibody. VAR2 protein was only detected in the fractions around 350kD.
B. VAR2 protein was immunoprecipitated with or without ATP under native conditions using VAR2 antibody and protein A-agarose. The precipitated proteins were run on a SDS-PAGE gel and silver stained.

FtsH1 antisense construct

Besides VAR2, there are three other FtsH-like in Arabidopsis. Among these three, FtsH1 is the only one has been proven to be located on the thylakoid membranes of chloroplasts (Lindahl et al, 1996). Based on the sequence similarities between VAR2 and FtsH1, it is possible that FtsH1 can compensate, at least in part, for a lack of VAR2 function, leading to the formation of normal green tissue in *var2*. To test this hypothesis, we made a FtsH1 antisense construct and transformed wild-type Col-0 and *var2-1* using *Agrobacterium*-mediated transformation method. All of the T1 transformed plants had the parental phenotype, suggesting that the FtsH1 antisense constructs neither can make wild type yellow nor can it make the phenotype of *var2-1* more severe. Of course, the FtsH1 expression levels in these antisense plants have to be determined in the future. Hopefully, one of them will have very low level of FtsH1 expression. Otherwise, a FtsH1 knock-out mutant has to be isolated in order to draw a final conclusion about whether FtsH1 is the redundent function for VAR2 or not.

Generation of *var2* suppressors

In order to study VAR2 redundant pathways, regulatory mechanisms, and interacting proteins, we used a genetic approach to generate *var2* suppressor mutants. This experiment was started before the gene was cloned. We chose *var2-4*, one of the most severe alleles, to do the experiments. EMS mutagenesis was performed on *var2-4* seeds. Green plants were found in the M1 plants. These green plants were transplanted to individual pots and their seeds were collected separately for further analysis. Seeds from the rest of the plants were

pooled together and planted again to screen for M2 (green) suppressor plants. M2 green plants were transplanted to individual pots, and their seeds were saved separately.

DISCUSSION

VAR2 protein complex

From *E. coli* FtsH to yeast YTA10-12, all members of the metallopeptidase subfamily of AAA ATPases form homo-oligomers (Neuwald et al., 1999). Results shown here suggest that VAR2 also functions as an oligomer. Both *E. coli* expressing VAR2 and Col-0 transformed with a VAR2-GFP construct exhibit dominant negative phenotypes, which suggests that VAR2 forms homo-oligomers *in vivo*. In *E. coli*, VAR2 serves as a mutant FtsH due to its sequence diversity; it likely poisons the function of native FtsH by forming non-functional hetero-oligomers. This phenotype is consistent with the dominant negative phenotype of *E. coli ftsH* mutations at the cytoplasmic domain, and indicates that VAR2 could oligomerize in the same manner as FtsH. The dominant negative effects of the VAR2-GFP construct in wild type Arabidopsis further implied that VAR2 forms homo-oligomers. Under native conditions, VAR2 eluted through gel filtration columns as a complex of about 350kDa. When immunoprecipitation experiments were conducted under the same conditions, VAR2 was the major band along with a minor and slightly bigger band. This proves that VAR2, indeed, forms homo-oligomers. The extra band might represent one or more interacting proteins. This interacting proteins could be a modulator of VAR2 activity just like HflK/C and YccA for FtsH in *E. coli* (Kihara et al., 1996). The interacting protein also could be FtsH1 of Arabidopsis in the same manner as the YTA10-12 complex, in which

YTA10 homo-oligoers and YTA12 homo-oligoers associate with each other (Arlt, et al. 1996). However, preliminary results from the FtsH1 antisense experiments indicate that changes in FtsH1 abundance do not affect the phenotype of Col-0 and *var2*, meaning that FtsH1 might not serve the same physiological function as VAR2. Of course, further characterization of the FtsH1 antisense plants is needed to confirm this conclusion.

Functions of VAR2

Increasing evidence indicates that AAA metalloproteases have two functions: chaperone-like activities and protease activities for specific substrates (Gottesman et al., 1997; Suzuki et al., 1997; Neuwald et al., 1999). To detect the protease activity of VAR2, *in vitro* translated VAR2 proteins were incubated with potential substrates. It is well known that SecY (a component of protein translocator complexes) is a substrate of FtsH in *E. coli* (Akiyama, et al. 1996). To test whether VAR2 has similar activity, we obtained the cpSecY, which is the Arabidopsis chloroplast homologue of the *E. coli* SecY, from Dr. Neil Hoffman's lab at Stanford. The *in vitro* translated VAR2 and cpSecY (^{35}S labled) were mixed and incubated at 37°C for 2 hours. No degradation of cpSecY was detected (assay conditions was described in Kihara et al. 1996). Two more general and commercially-available substrates (resorufin-labeled Casein from Boehringer Mannheim and PepTag from Promega) were used for VAR2 protease detection, but neither of them was digested by VAR2.

From the negative results, it cannot be concluded that VAR2 has no protease activity. First, FtsH-like metallopeptidases are not general proteases but, rather, they are specific for selective substrates, so it could be that none of the substrates I tried fit the characteristics of

its native substrates. Secondly, VAR2 forms oligomers and interacts with other proteins, and it might be that the protease activity of VAR2 requires a certain kind of protein complex that cannot be formed *in vitro*. Another possibility could be simply that we could not get enough of VAR2 from the *in vitro* translation reactions for these kinds of protease assays. In other words, there may have been too little protease activity to detect a decrease in labeled SecY or in the other potential substrates. For example, when *E. coli* FtsH was tested for its protease activity toward SecY, a larger amount of protein was used than in the present experiments (Akiyama et al., 1996). The problem with VAR2 is that it cannot be over-expressed in *E. coli*, because *E. coli* that over-express VAR2 have the filamentation phenotype and die. Therefore, it might be necessary to try other expression systems to over-express VAR2 in the future.

Given the predicted function of VAR2, how can we relate such a chaperone/protease to thylakoid membrane biogenesis? To make the connection, we have to discuss PFTF, which is the most homologous FtsH-like protein to VAR2. PFTF was isolated using a biochemical assay as a protein required for fusion of vesicles from red pepper chromoplasts (Hugueney, et al. 1995). As discussed in chapter 3, in the past decades accumulating microscopic evidence has demonstrated that vesicular movements are involved in inner plastidic membrane biogenesis (Morre, et al. 1991). In the transition processes between different plastid types, it is thought that vesicles, moving between the internal plastid envelope and inner plastid membrane systems, serve as vehicles to transport different kinds of lipids, proteins, and pigments. In the transition from proplastid to chloroplast during early leaf development, the thylakoid membrane may be built up by the fusion of vesicles derived from the inner chloroplast membrane. With 78% identity in amino acid sequence between

PFTF and VAR2, it is reasonable to suggest that VAR2 is required for vesicle fusion events in early chloroplast differentiation similar to PFTF. The totally yellow young leaves of *var2* also indicate that VAR2 is crucial at an early stage of chloroplast development.

To investigate VAR2 function in thylakoid membrane biogenesis, it is important to hunt for VAR2 substrates and to characterize the function of the substrates. However, as we learned from *E. coli*, FtsH has substrates in different physiological pathways, and not every substrate is invovled in filamention. The same scenario might apply to VAR2. So, even if we find out some substrates of VAR2, they might not be directly involved in the variegation phenotype. Thus, we may have to sort out those substrates that are related to the variegation phenotype and chloroplast differentiation. The *var2* suppressor analyses would be a powerful tool to isolate components and pathways directly related to the variegation phenotype, simply because we are looking for green suppressor plants. One thing I have to point out here is that *var2-4* was used in generating suppressors in my experiments. Since we already know that *var2-4* is a null allele, the suppressors we are expecting to obtain would be in the redundant pathway(s) rather than directly interacting proteins. Therefore, it would be valuable to generate and isolate other kinds of suppressors from weak mis-sense alleles like *var2-3* or *var2-5* to isolate directly interacting proteins in the same pathway.

Allelic series for VAR2 structure and function studies

All FtsH-like proteins have three distinctive domains, including the AAA cassette, the zinc binding site, and the region between the two transmembrane segments (Patel and Latterich 1998). The AAA cassette contains the Walker A and B ATP binding sites. AAA cassette of YME has recently been demonstrated to have chaperone activity of binding to

denatured proteins (Leonhard et al., 1999). The zinc binding site is conserved among metalloproteases and is apparently required for protease activity. The region between the two transmembrane segments appears to be involved in the oligomerization of FtsH monomers (Akiyama et al., 1998). As we can see, the functions of these domains have been extracted from results of FtsH-like proteins from different sources.

The *var2* allelic series provides us a good opportunity to investigate the function of each domain and the relationship among these domains in VAR2. Obviously, null alleles (*var2-1*, *var2-4*, and *CS3166*) would not be valuable for such experiments, since there would not be any expression of the mutant gene products. The rest of the alleles can be divided into three categories based on the location of the mutations. Both *var2-3* and *var2-5* are mutations in the ATP binding sites; *var2-2* is a missense mutant at the end the second transmembrane segment; and CS3622 has a piece of polypeptide inserted into the C-terminus (presumably the protease domain) due to skipping of the last intron. Hopefully, more mis-sense alleles can be identified from the rest of the *var2*-like mutant lines to enlarge the collection of each category. The first question to be answered is whether some of these alleles are dominant alleles in the same manner as the VAR2-GFP construct. Since these alleles do have mutant protein expressed (data not shown), a dominant phenotype would indicate that the mutant VAR2 protein could still form oligomers with native VAR2; a recessive phenotype would suggest that the oligomerization capacity was abolished by the specific mutation. The oligomerization state of VAR2 can also be assessed in these alleles by biochemical means such as immunoprecipitation and gel filtration chromatography. In this way, we can answer more questions, such as whether ATP or zinc is required for oligomerization of VAR2, whether oligomerization is an absolute prerequisite for VAR2

protease activity, and whether any of these domains is required for interacting with other proteins.

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CHAPTER 5. SUMMARY

In higher plants, plastid development and differentiation are regulated by both environmental and developmental stimuli through the interplay of nuclear and chloroplast factors. Under normal growth conditions, undifferentiated proplastids in meristem cells differentiate into different types of plastids, such as chloroplasts in mesophyll cells, amyloplasts in root cells, and chromoplasts in fruit and flower. In dark grown plants, mesophyll cells develop etioplasts, and etioplasts will differentiate into chloroplasts after the plants are transferred into light. In senescent leaf cells, chloroplasts differentiate into chromoplasts. The conversion of one of plastid into another usually involves massive construction or reconstruction of plastid inner membrane structures, for example, the conversion of crystalline prolamellar bodies in etioplasts into thylakoids in chloroplasts. Inner membrane structures are not static, either, and both protein components and lipid contents are constantly modified. All of these changes in plastids are highly regulated by the nuclear genome, but the mechanisms governing plastid membrane biogenesis are poorly understood. We characterized the *var2* variegation mutant to gain insight into the mechanism involved in thylakoid membrane biogenesis.

The *var2* mutant of Arabidopsis has striking bright yellow emerging first leaves and green and white (or yellow) variegated mature first and subsequent leaves. While chloroplasts in the green cells are normal, the plastids in the white or yellow cells lack organized internal thylakoid membrane structures. All of the green tissues of the *var2* plant, except the cotyledons, are variegated. The degree of variegation is generally not affected by light or temperature, which indicates that the disruption of thylakoid membrane structure is not due to photooxidation. However, *var2* leaves do become less variegated under some

conditions, such as dim light (10 to 15 μE) or higher temperature (above 28°C), in which plant growth rate is slowed down (Chen et al., 1998). During early leaf development, chloroplasts undergo fast division and increase in number from 10-20 per cell to 100-150 per cell (Mullet, 1988). The fact that a slow growth rate can make *var2* emerging leaves almost all green, as opposed to all yellow under normal growth rates, suggests that the *var2* gene product is required for thylakoid membrane biogenesis during early chloroplast differentiation when there are rapid chloroplast divisions.

The *var2* mutant was first mapped on chromosome II by Martínez-Zapater (Martínez-Zapater, 1993). We further mapped and cloned the *VAR2* gene. The deduced amino acid sequence of *VAR2* shares significant similarity with *E. coli Fts*H and *Fts*H homologues from other species including PFTF (protein fusion and translocation factor) from red pepper (Hugueney et al., 1995), an *Fts*H homologue of *Arabidopsis* (*Fts*H1)(Lindahl et al., 1996), and four *Fts*H-like ORFs of *Synechocystis sp.* PCC 6803. *Fts*H belongs to a novel family of ATPase called AAA-ATPase (ATPases associated with diverse cellular activities). These proteins are found in both eukaryotes and prokaryotes and mediate various cellular processes, including vesicle-mediated membrane fusion, organelle biogenesis, protein translocation, and protein degradation (Beyer, 1997; Patel and Latterich, 1998). VAR2 protein, like other FtsH-like proteins, has two transmembrane domains in its N-terminus, ATPase conserved domains including the Walker ATP binding site A and B, and a zinc protease domain at its C-terminus. The chloroplast import experiments suggest that VAR2 is localized on the thylakoid membrane with its C-terminal end facing the stroma. Phylogenetic analysis shows that *VAR2* is closely related to PFTF and that there are two groups of FtsH-

like proteins in photosynthetic organisms. VAR2 is in a different lineage than the other Arabidopsis FtsH-like proteins, suggesting that it may have very different functions.

The structure and function properties of *Fts*H-like proteins are best characterized in *E. coli* and yeast. FtsH in *E. coli* has been shown to be a membrane-bound zinc dependent protease that forms a complex with HflK/C and YccA to selectively degrade unassembled proteins (Tomoyasu et al., 1993; Tomoyasu et al., 1993). This is termed protein "quality control". Its substrates include SecY (Kihara et al., 1998; Kihara et al., 1996), which is a subunit of protein translocase of *E. coli*, heat-shock transcription factor σ^{32} (Herman et al., 1995), H^+ ATPase F_0 sector, (Akiyama et al., 1996), and CII, a transcription factor that determines the decision of lysis or lysogeny of phage lambda (Kihara et al., 1997). The function of FtsH is not limited to protein "quality control", and it is also involved in mRNA decay in *E. coli* and a chaperone-like activity associated with protein integration into membrane (Akiyama et al., 1994; Akiyama et al., 1994; Shirai et al., 1996; Suzuki et al., 1997). *E. coli* cells that expressed VAR2 protein exhibit a temperature sensitive filamentous phenotype at 42°C, which resembles dominant mutants of *E. coli* FtsH . It is tempting to suggest that VAR2 acts in a similar manner as FtsH in *E. coli* and VAR2 protein interacts with the native FtsH to give rise to the dominant negative phenotype. In support of this conclusion, the *in vitro* immunoprecipitation and gel filtration studies indicate that VAR2 forms homo-multimeric complexes.

As discussed in Chapter 4, VAR2 has two proposed functions, in accord with other FtsH-like proteins. On one hand, its AAA domain could have chaperone activity to facilitate protein complex formation or dissociation; on the other hand, its C-terminus might have metallopeptidase activity to degrade mis-assembled components of a protein complex. Also,

from studies on *E. coli* FtsH, we suspect that the substrates of VAR2 might be broadly represented. The *var2* variegation phenotype might be due to effects on one of the substrates of VAR2. There are two possibilities in terms of how VAR2 might be involved in thylakoid membrane biogenesis. First, VAR2 could be directly related to membrane fusion or fission events. It has been suggested that vesicles derived from the inner chloroplast membrane are involved in thylakoid membrane biogenesis. VAR2 could be responsible for facilitating certain complex formation or dissociation, which in turn is required in vesicular fusion events. Secondly, VAR2 could affect the formation or modeling of some general protein complex that is important in early chloroplast biogenesis, such as cpSecY. Apparently, without a proper cpSecY/E/A complex, which mediates protein translocation through the thylakoid membrane, thylakoid membranes are affected dramatically. For example, a SecY mutant in maize shows a similar phenotype to *var2* in terms of lacking organized chloroplast internal membranes (Roy and Barkan, 1998).

The current model of the mechanism of *var2* variegation is illustrated in Figure 1. From the phenotypes of *var2*, I hypothesize that VAR2 is involved in thylakoid membrane biogenesis either directly or indirectly. Also as discussed above, cell and/or chloroplast division rates affect the *var2* phenotype. So, I hypothesize that VAR2 is most needed at the early chloroplast differentiation stage, when cell and chloroplast divisions are at their fastest rates. The process that VAR2 facilitates can happen spontaneously, but slowly, or it can be partially replaced by another chaperone or protease, but in a slower process. This hypothesis can explain most of the phenotypes of VAR2. In dark grown plants, VAR2 message is not detected (data not shown), so the etioplasts in *var2* plants are normal. In the cotyledons of

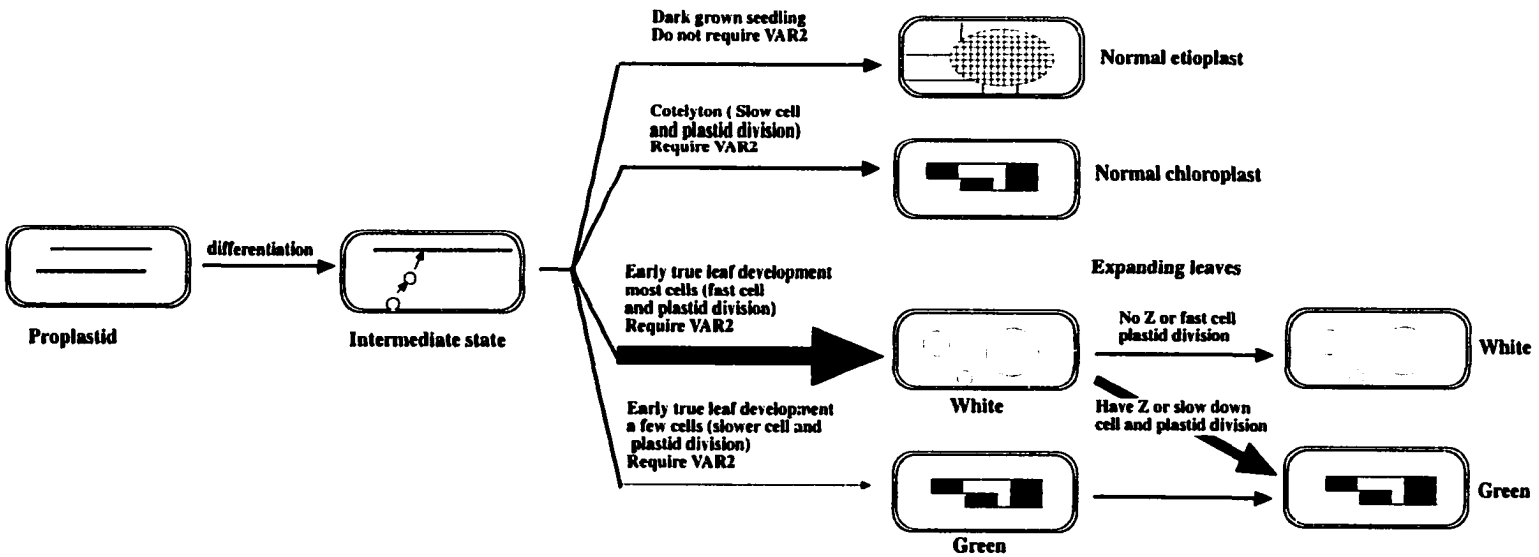

Figure 1. Model for *var2* variegation mechanism

var2 plants, since there is nearly no cell division in cotyledons, VAR2 activity could be compensated for by the slower spontaneous or redundant activities. In leaf development, we have to divide it into two stages. One stage is the early leaf development stage, when both cells and chloroplasts are dividing very fast; another is the leaf expansion stage, when cells mainly undergo expansion rather than division. During early leaf development, about 30 cells in the leaf primodium will undergo fast cell division to form a leaf. At the same time, chloroplasts have to go through fast division as well not only to keep up the fast cell division rate but also to increase the number of chloroplast per cell from about 20 to 150-200. In the absence of VAR2 activity, chloroplasts cannot develop normal thylakoid membrane, so what we see are yellow emerging first leaves. Of course, the cell and chloroplast division rate can not be absolutely uniform, there are always individual cells or chloroplasts dividing at a slower rate which leads to scattered tiny green spots on a yellow background. As the leaf reaches its expansion stage, cell and chloroplast division rates slow down, and the spontaneous and redundant activities kick in to create more and more green cells. This is why we see green sectors emerge and expand after the leaves reach a certain stage. There are always some cells that cannot turn green. I suspect that the conversion from white to green can only happen before a certain stage of either cell or chloroplast development. Once that stage is passed, abnormal plastids will be arrested in the "white state". In this way, it will always end in a variegation phenotype, simply due to differences in cell and/or chloroplast division rates among the cell population.

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